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UNIVERSITY OF ALBERTA

Functional and Molecular Studies of Eucaryotic Nucleoside Transporters

BY



Douglas L. Hogue

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for a degree of Doctor Of Philosophy.

Department of Biochemistry

Edmonton, Alberta

Fall, 1994



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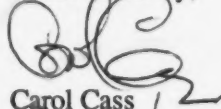
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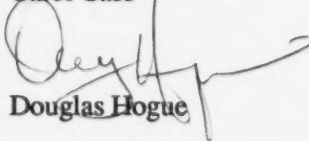
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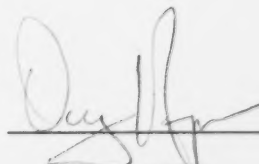
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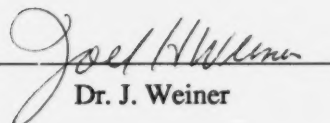
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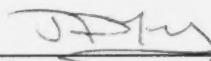
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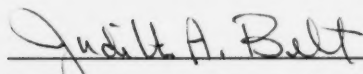
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One's accomplishments reflect those of the individuals that most shaped that person.

Hence, this is dedicated to Gwen and Leon Hogue,

Kathleen Eberly McNair,

and especially, Calla Shank-Hogue.

ABSTRACT

Flux of physiological nucleosides and some pharmacologically relevant nucleoside analogs across the plasma membrane of mammalian cells occurs primarily via translocation events that are mediated by nucleoside-selective transport (NT) processes that function by equilibrative or concentrative mechanisms. Equilibrative NT processes are differentiated into NBMPR-sensitive or NBMPR-insensitive NT processes based upon their sensitivity to inhibition by nanomolar or micromolar concentrations of NBMPR, respectively. Na^+ /nucleoside (concentrative) co-transport processes are primarily differentiated on the basis of permeant selectivity. The function of these NT processes is well documented, however little is known of the structure of the polypeptides responsible. The role of glycosylation in the functional expression of NT proteins and the identity of cDNA that encode NT polypeptides has been investigated.

Inhibition of asparagine-linked glycosylation in mouse L1210/C2 cells by exposure to tunicamycin altered the kinetics of the low-affinity NT process and induced changes in concentrative and NBMPR-sensitive NT activities. Intracellular retention of NBMPR-sensitive NT polypeptides was also observed. The asparagine-linked oligosaccharide of the NBMPR-sensitive NT polypeptides were of a multiple antennary complex and/or hybrid type.

The high-affinity pyrimidine- and purine-selective NT processes of *Spodoptera frugiperda* (Sf9) cells were characterized in an effort to identify host cells for use in an expression cloning method. These NT processes functioned by an equilibrative mechanism, were insensitive to inhibitors of mammalian NT and appeared to be distinct from previously described eucaryotic NT processes.

A unique mammalian NT protein was isolated by a functional expression cloning method in *Saccharomyces cerevisiae*. The capacity of the transporter protein to mediate nucleoside flux was confirmed by its functional expression in *Xenopus laevis* oocytes and its localization to an intracellular membrane, likely Golgi, was demonstrated in immunological experiments.

In summary, this investigation has demonstrated a role for N-linked glycosylation in the functional expression of NT processes and has revealed the existence of novel NT processes in the plasma membranes of Sf9 cells and identified a NT protein in the Golgi membranes of mammalian cells.

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ABBREVIATIONS

$\bar{\epsilon}$	Molar extinction coefficient
Ade	Adenine
Ado	Adenosine
ATCC	American Type Culture Collection
B _{max}	Maximum number of ligand binding sites per cell
bp	Nucleotide basepair
BSA	Bovine serum albumin
BTB	Bug transport buffer
cDNA	Complementary DNA
cRNA	Complementary RNA
<i>ci</i> NT	Concentrative, NBMPR-insensitive nucleoside transport
<i>cib</i> NT	Concentrative, NBMPR-insensitive, broad permeant selectivity nucleoside transport
<i>cif</i> NT	Concentrative, NBMPR-insensitive, formycin-B selective nucleoside transport
<i>cit</i> NT	Concentrative, NBMPR-insensitive, thymidine selective nucleoside transport
Cyd	Cytidine
Cyt	Cytosine
dAdo	2'-Deoxyadenosine
DEAE	Diethylaminoethyl
dH ₂ O	Distilled H ₂ O
Dilazep	3,4,5-Trimethoxybenzoic diester with tetrahydro-1H-1,4-diazepine-1,4(5H)-dipropanol
DTT	Dithiothreitol
dUrd	2'-Deoxyuridine
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
<i>ei</i> NT	Equilibrative, NBMPR-insensitive nucleoside transport
<i>es</i> NT	Equilibrative, NBMPR-sensitive nucleoside transport
ER	Endoplasmic reticulum
FTM	Fishers transport medium
Guo	Guanosine

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
IC ₅₀	Concentration of drug inhibiting the parameter measured by 50%
Ino	Inosine
K _d	Dissociation constant
kDa	kilodaltons
K _m	Permeant concentration at half-maximal unidirectional flux
λ _{max}	Wavelength of maximum absorbance
M _r	molecular weight
MTP	Mouse Transporter Protein
MTX	Methotrexate; 4-amino-10-methylfolic acid
NBMPR	Nitrobenzylthioinosine; 6-[(4-nitrobenzyl)-thio]-9-β-D-ribofuranosylpurine
NBTGR	Nitrobenzylthioguanosine; 2-amino-6-[(4-nitrobenzyl)-thio]-9-β-D-ribofuranosylpurine
NMG	N-methyl-D-glucammonium
NT	Nucleoside transport
nt	Nucleotides
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCA	Perchloric acid
pfu	Plaque-forming units
PMSF	Phenylmethylsulfonyl fluoride
RT	Room temperature
RTM	RPMI 1640 transport medium
SD	Standard deviation
SAA	Sulfanilamide; p-aminobenzenesulfonamide
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis
SDS	Sodium dodecylsulphate
TBS	Tris-buffered saline
TFMS	Trifluoromethane sulfonic acid
TLC	Thin layer chromatography
TK	Thymidine kinase
Tris	Tris-(hydroxymethyl) aminomethane
UDP	Uridine-5'-diphosphate

UMP	Uridine-5'-monophosphate
Ura	Uracil
Urd	Uridine
UTP	Uridine-5'-triphosphate
UV	Ultraviolet
UXP	5'-phosphorylated uridine

Chapter I

GENERAL INTRODUCTION

Flux of physiological nucleosides across the plasma membrane of mammalian cells occurs primarily via translocation events that are mediated by nucleoside-selective transport (NT) processes. The relatively hydrophilic nature of nucleosides precludes, for the most part, a significant contribution to flux by passive diffusion across the lipid bilayer of the plasma membrane. Although some cell types lack the capacity for *de novo* biosynthesis of purines (Murray, 1971), most mammalian cell types are able to synthesize purine and pyrimidine nucleotides by *de novo* pathways and the presence of NT processes is thus not a strict necessity. However, the capacity of NT processes to provide metabolically "less-expensive" nucleotide precursors than the *de novo* nucleotide biosynthetic pathways is likely exploited by cells. The luminal epithelial cells of the intestine and kidney play an important role in nucleoside salvage, acting to obtain nucleosides from dietary sources and body fluids.

Perhaps more importantly, NT processes act to modulate local concentrations of extra- and intracellular pools of adenosine. Extracellular adenosine, which exhibits a short half-life due to rapid metabolism after release from cells, acts as a local metabolic regulator by binding to adenosine receptors and subsequently eliciting a variety of physiological reactions (Stiles, 1992). Many organs are targets of adenosine receptor activation, which in turn modulates such diverse physiological functions as smooth muscle tone, cardiac contractility, renal function, lipolysis, brain ischemia, and release of neurotransmitters (Ramkumar *et al.*, 1988; Rudolphi *et al.*, 1992). The rapid creation and depletion of the extracellular pools of adenosine involved in the stimulation and inactivation of adenosine receptors, respectively, are thought to be due, in part, to fluxes mediated by NT processes.

Binding of UTP and ATP to receptors distinct from the adenosine receptors also modulates physiological activities in a variety of cell types (O'Conner, 1992). The regulation of UTP/ATP receptor activity resides in the capacity of 5'-ectonucleotidases to hydrolyze nucleotides (Zimmermann, 1992), an event that is likely followed by uptake of extracellular adenosine and uridine via NT processes. Indeed, the presence of a functional link between 5'-ectonucleotidase activity and a NT process has been demonstrated in hepatocytes (Che *et al.*, 1992). The role of NT processes therefore appears to be multi-faceted, including regulation of ligand-receptor interactions that mediate important physiological functions as well as providing a means by which to obtain purine and pyrimidine nucleosides from extracellular fluids and dietary sources.

Nucleoside Transport is Mediated by a Family of Functionally Distinct Processes

Functional studies conducted with cultured cell lines and isolated cells and tissues have revealed that NT processes can be categorized into either of two mechanistic families: facilitated diffusion (equilibrative) transporters and Na⁺/nucleoside (concentrative) co-transporters¹. By definition, equilibrative transporters act to translocate a permeant² down its transmembrane concentration gradient and are characterized by saturation of transport rate as a function of permeant concentration and competition of structurally related permeants for the translocation process (Stein, 1986). Co-transporters couple the transmembrane movement of a small molecule down its concentration gradient to that of the permeant, thereby leading to unidirectional flux of both types of molecules. This mechanism allows for transport of a permeant against its own transmembrane gradient.

Members within each of the mechanistic families of nucleoside transporters have been further categorized based upon their permeant selectivities, kinetic properties,

¹Throughout this study, the facilitated diffusion and Na⁺/nucleoside co-transporter processes are referred to as the equilibrative and concentrative NT processes, respectively.

²The term "permeant" refers to a molecule that is translocated across the membrane barrier. This definition excludes structurally similar molecules that may interact with the transporter but that are not translocated.

sensitivities to inhibitory compounds and, in the case of concentrative transporters, the Na^+ /nucleoside coupling stoichiometry. That most cultured cell types and tissues simultaneously co-express two or more mechanistically distinct NT processes as a "heterogeneous" NT phenotype has complicated attempts to identify and fully characterize each transport process.

Two Distinct Equilibrative NT Processes Exhibit Different Sensitivity to Inhibition by Nitrobenzylmercaptapurine Riboside (NBMPR)

NBMPR, a *p*-nitrobenzylthiopurine nucleoside originally described by Paul *et al.* (1975), has been used to identify and differentiate between two equilibrative NT processes expressed in most, if not all, mammalian cells (For reviews, see: Cass *et al.*, 1987; Plagemann *et al.*, 1988; Gati and Paterson, 1989). The function of equilibrative NBMPR-sensitive (*es*) NT processes is totally inhibited in the presence of ≤ 10 nM NBMPR, whereas that of equilibrative NBMPR-insensitive (*ei*) NT processes is not inhibited at these low NBMPR concentrations, but rather at μM concentrations. A number of other structurally unrelated compounds, including dipyrindamole and dilazep, are also inhibitors of both the *es* and *ei* NT processes (Paterson *et al.*, 1983).

Although nucleosides accepted as permeants of the two equilibrative NT processes may differ widely in the configuration of their purine or pyrimidine base structures, each process exhibits a distinct stereoselectivity for the naturally occurring D-enantiomeric, as opposed to L-enantiomeric, configuration of ribofuranosyl moiety (Gati *et al.*, 1989; Paterson *et al.*, 1991). The 5'- and 3'-hydroxyl groups of the ribofuranosyl moiety are evidently important for recognition by the *es* transporter, based upon results of permeant-competition studies conducted in erythrocytes and L1210/B23 cells (Cass and Paterson, 1972; Gati *et al.*, 1984; Vijayalakshmi *et al.*, 1994a). The latter is a mutant L1210 cell line that expresses only *es* NT (Vijayalakshmi *et al.*, 1992). Several nucleoside derivatives have been directly demonstrated to not be permeants or to be very poor permeants of the *es* NT process including 2',3'-dideoxyadenosine (ddA; Plagemann and Woffendin, 1989a), 2',3'-dideoxyguanosine (ddG; Gati *et al.*, 1992), 2',3'-dideoxycytidine (ddC; Plagemann and Woffendin, 1989b), 2',3'-dideoxythymidine (ddT; Domin *et al.*, 1988), 2',3'-didehydro-3'-deoxythymidine (d4T; August *et al.*, 1991), and 3'-azido-3'-deoxy-thymidine (AZT; Zimmerman *et al.*, 1987). A permeant-competition study has shown that the 3'-hydroxyl group, but not the 5'-hydroxyl group, is necessary for recognition of permeant by the *ei* transporter of rat Walker 256 cells (Vijayalakshmi *et al.*, 1994b). Thus, it appears that differential hydrogen bonding of ribofuranosyl hydroxyl groups to permeant binding site may occur between the *es* and *ei* NT transporters. This suggestion is supported by the observation that *ei* NT processes tend to exhibit much lower permeant affinities (K_m values: high μM to mM concentrations) than *es* NT processes, which generally exhibit K_m values of ≤ 300 μM (Plagemann *et al.*, 1988; Boleti, 1991).

The human erythrocyte expresses only the *es* NT process (Cass and Paterson, 1972; Cass *et al.*, 1974), and as such, has been extensively used for much of the functional characterization of the *es* NT process. The kinetic behavior of the process mediating uridine transport in erythrocytes, as measured under zero-*trans*³, infinite-*cis* and equilibrium exchange conditions, conforms to a simple carrier model of facilitative diffusion (Cass *et al.*, 1974; Cabantchic and Ginsburg, 1977; Wohlheuter and Plagemann, 1982; Jarvis *et al.*, 1983a). The *es* nucleoside transporter also displays a differential translocation mobility in the loaded and empty states (Wohlheuter and Plagemann, 1982; Jarvis *et al.*, 1983a; Plagemann and Wohlheuter, 1984). A detailed

³The *cis* face of the plasma membrane denotes the face from which transport is being measured, therefore, zero-*trans* transport refers to transport of permeant from one face (ie. *cis*) of the membrane to the other (ie. *trans*) as measured when the concentration of permeant at the *trans* face is zero (Stein, 1986).

kinetic characterization of NT processes of human erythroleukemia K562 cells, which express both *es* and *ei* NT processes, has been performed (Boleti, 1991). This kinetic study, conducted under zero-*trans* conditions in the presence of low concentrations of NBMPR (to eliminate flux mediated by the *es* NT process), demonstrated that the *ei* NT process exhibits properties typical of a facilitative diffusion carrier.

The ability of low concentrations (≤ 10 nM) of NBMPR, and other *p*-nitrobenzylthiopurine nucleoside analogs (Paterson *et al.*, 1983), to totally inhibit nucleoside flux is directly correlated with the presence of high-affinity NBMPR binding sites (K_d , 0.1-2 nM) in the plasma membrane of erythrocytes (Cass *et al.*, 1974; Jarvis *et al.*, 1982) and cultured cells (Cass *et al.*, 1981). Occupancy of these sites is competitively inhibited by purine and pyrimidine nucleosides (Cabanchik *et al.*, 1977; Jarvis *et al.*, 1983a; Jarvis *et al.*, 1983b). The demonstration of NBMPR binding sites and NBTGR inhibition of nucleoside permeation in reconstituted preparations of purified human erythrocyte NT protein provided direct evidence for a functional relationship between the high-affinity NBMPR binding sites and the *es* NT protein (Kwong *et al.*, 1988). The ability of NBMPR to competitively inhibit influx and non-competitively inhibit efflux of nucleosides (Cabanchik *et al.*, 1977; Jarvis *et al.*, 1983a), combined with the observation of greater NBMPR binding at 4°C to right side-out versus inside-out vesicles of erythrocyte plasma membranes (Agbanyo *et al.*, 1988), indicates that NBMPR binds to the extracellular face of the *es* NT protein. Quantitation of NBMPR binding sites under equilibrium conditions has revealed that *es* NT-expressing cell lines typically exhibit 10^3 - 10^5 sites per cell. An exception is the human BeWo choriocarcinoma, for which cells have been reported to exhibit as many as 27×10^6 sites per cell (Boumah *et al.*, 1992).

While a direct relationship between the presence of high-affinity NBMPR binding sites and *es*-type NT is indisputable in erythrocytes and many cultured cell types, exceptions have been observed. Several mutants of the mouse S49 lymphoma cell line, the cells of which normally express only *es* NT activity, possess wild-type numbers of NBMPR binding sites but express NBMPR-insensitive NT activity (Aronow *et al.*, 1985). The presence of NBMPR binding sites has been reported in the Novikoff hepatoma, although NT activity in cells of this line show little or no sensitivity to inhibition by nanomolar concentrations of NBMPR (Plagemann and Wohlheuter, 1985; Gati *et al.*, 1986). Thus, the relationship between the NBMPR binding and permeant binding sites of the *es* NT protein is not clearly understood.

In the case of the *es* NT process, dipyrindamole competitively inhibits both binding of NBMPR to its high-affinity sites and nucleoside influx, whereas nucleoside efflux is inhibited in a non-competitive manner (Jarvis *et al.*, 1986; Paterson *et al.*, 1980). This suggests a direct interaction of dipyrindamole with the NBMPR binding site. Similarly, inhibition of high-affinity NBMPR binding by dilazep is competitive (Gati and Paterson, 1989). Little is known of the mechanism of interaction of the inhibitors dilazep and dipyrindamole with the *ei* NT process. Low-affinity binding of dipyrindamole to sites distinct from high-affinity NBMPR binding sites has been observed (Jones and Hammond, 1992), although attempts to demonstrate specific binding of dipyrindamole to sites associated with the *ei* NT process have been unsuccessful (Shi and Young, 1986; Woffendin and Plagemann, 1987).

Structural Characteristics of the Equilibrative NBMPR-Sensitive (*es*) NT Protein

The identification and isolation of *es* NT proteins has been possible because of the highly selective interaction of NBMPR with the *es* NT protein. Direct identification of *es* NT proteins in plasma membranes with [^{3} H]NBMPR and [^{3} H]N⁶-(*p*-aminobenzyl)-adenosine was achieved by covalently labeling to their binding site through an UV-induced photoreactive mechanism (Young *et al.*, 1983; Young *et al.*, 1984). The presence of a photoreactive sulfur-benzyl bond in NBMPR, coupled with the inability of

pre-photolysed [^3H]NBMPR to form covalent linkages with the *es* NT protein, suggests that NBMPR itself is the photoreactive species (Jarvis and Young, 1987; Fleming *et al.*, 1990).

The *es* NT protein in the plasma membranes of human erythrocytes and some cultured cell types has been identified by [^3H]NBMPR-photolabeling as a 50-60 kDa protein on SDS-polyacrylamide gels (Young *et al.*, 1983; Young *et al.*, 1984; Jarvis and Young, 1987; Izerman *et al.*, 1989; Hogue *et al.*, 1990; Boumah *et al.*, 1992; Kwong *et al.*, 1993). Exceptions to these observations have been: pig erythrocyte, 64 kDa (Kwong *et al.*, 1986); Novikoff hepatoma cells, 75 kDa (Gati *et al.*, 1986); human CCRF-CEM cells, 75 kDa (Crawford *et al.*, 1990a); and Erlich ascites cells, 42 kDa (Hammond and Johnstone, 1989). It appears that a differing extent of N-linked glycosylation accounts for the majority of molecular weight differences of [^3H]NBMPR-photolabeled proteins from different cell types, since digestion of plasma membrane preparations with endoglycosidase F (or peptide-N-glycosidase F) generates an ≈ 40 -45 kDa [^3H]NBMPR-photolabeled protein (Kwong *et al.*, 1986; Hogue *et al.*, 1990; Jarvis and Young, 1987; Kwong *et al.*, 1993). The 42 kDa [^3H]NBMPR-photolabeled protein of Erlich ascites cells apparently contains little or none N-linked oligosaccharide (Hammond and Johnstone, 1989). Alternatively, the pig erythrocyte [^3H]NBMPR-photolabeled protein is reduced to only 57 kDa upon removal of N-linked oligosaccharide (Kwong *et al.*, 1986; Good *et al.*, 1987), suggesting the *es* NT protein is larger in pig erythrocytes compared to other cell types.

A structural relatedness of *es* NT proteins from different cell types is suggested by results of two studies that employed complementary methods. Firstly, trypsin digestion of the deglycosylated [^3H]NBMPR-photolabeled proteins of human erythrocytes, rat liver, and guinea pig liver gave rise to near-identical tryptic fragmentation patterns (Kwong *et al.*, 1993). Secondly, polyclonal antibodies raised against purified human erythrocyte *es* NT protein recognized the *es* NT proteins of rat liver cells and pig and rabbit erythrocytes (Kwong *et al.*, 1992). These studies suggest that *es* NT proteins of different species share common elements of amino acid structure.

The structural relationship between the *es* and *ei* NT proteins is uncertain. A difference in protein structure is suggested by the observation that these two NT proteins exhibit differing sensitivity to inhibition by the sulfhydryl-reactive agent *p*-chloromercuriphenyl sulfonate (*p*CMBS). The *ei* NT process is inhibited by *p*CMBS with an IC_{50} value⁴ of 25 μM whereas ≥ 500 μM *p*CMBS was required for inhibition of the *es* NT process (Belt and Noel, 1985; Jarvis and Young, 1986). Furthermore, evidence that the *es* and *ei* NT proteins, as well as the proteins that mediate concentrative NT, are products of separate genes is provided by the isolation of NT-defective mutants from L1210 cells. Mouse L1210 cells normally express a concentrative (*cif*) NT process, in addition to the *es* and *ei* NT processes (Dagnino, 1988; Dagnino and Paterson, 1990; Dagnino *et al.*, 1991a). A mutant L1210 cell line (L1210/MC5-1 cells) lacking the parental *ei* NT process was isolated after a single-step mutagenesis event (Belt and Noel, 1988). When L1210/MC5-1 cells were further subjected to single-step mutagenesis, L1210/MA-27 cells, which expresses only the concentrative NT process, were isolated (Crawford *et al.*, 1990b). The capacity to selectively eliminate individual processes from the repertoire of NT processes normally expressed by L1210 cells suggests that each arises as distinct gene products. However, the exact relationship between equilibrative NT proteins of different species or tissues and their relatedness to other permeant transporters remains unknown since an amino acid or cDNA sequence of an equilibrative-type nucleoside transporter has not yet been identified.

⁴The concentration of compound inhibiting the parameter measured by 50%.

Mediated Nucleoside Flux by Functionally Distinct Concentrative Nucleoside Transport Processes

To date, four distinct concentrative NT processes have been identified by their capacity to mediate Na^+ -dependent nucleoside fluxes. These concentrative transporters may be differentiated on the basis of permeant selectivity, Na^+ /nucleoside coupling stoichiometry, and their sensitivity to inhibition by NBMPR. Terminology for the concentrative NT processes was originally proposed by Vijayalakshmi and Belt (1988) and was based upon the concentrative NBMPR-insensitive (*ci*) nature of the NT processes identified at that time. The *ci* NT processes may be further distinguished based upon their permeant selectivity, as discussed below.

The existence of *ci* NT processes that accept both pyrimidine and purine nucleosides as permeants has been demonstrated in rabbit choroid plexus (Wu *et al.*, 1992), rat and rabbit jejunum (Huang *et al.*, 1993; Terasaki *et al.*, 1993), and rabbit and human kidney (Guteierrez *et al.*, 1992; Pajor and Wright, 1992; Guteierrez and Giacomini, 1993; Brett *et al.*, 1993). These *ci* broadly permeant-selective (*cib*) NT processes exhibit apparent K_m values of ≤ 50 μM , as determined by measurement of zero-*trans* nucleoside influx and of nucleoside influx in the presence of competing nucleosides (IC_{50} values). In a detailed study by Wu and co-workers, the *cib* NT process was shown to (i) exhibit a stoichiometric coupling ratio between Na^+ and nucleoside of 2:1, and (ii) not accept 2',3'-dideoxyadenosine or 2',3'-dideoxycytidine as permeants (Wu *et al.*, 1992).

Two other distinct *ci*-type NT processes, each accepting both uridine and adenosine as permeants, have been identified and differ from *cib* NT processes in having narrower permeant selectivities. The *cit* NT process accepts primarily pyrimidine nucleosides as permeants, hence the designation *ci* thymidine-selective (*cit*). In addition, a *ci* NT process that accepts primarily purine nucleosides as permeants has been identified from its ability to mediated the influx of formycin B, a non-metabolized C-glycoside analogue of inosine. This process has been designated the *ci* formycin B-selective or *cif* NT process. An alternative nomenclature for these NT processes has been employed by Williams and Jarvis (1991) and Huang *et al.* (1993) wherein the *cif*, *cit* and *cib* processes are referred to as N1, N2 and N3, respectively. In contrast to the *cib* NT process, a stoichiometric coupling ratio between Na^+ and nucleoside of 1:1 has been observed with the *cif* (Dagnino *et al.*, 1991b; Plagemann and Aran, 1990a; Plagemann *et al.*, 1990) and *cit* NT processes (Jarvis *et al.*, 1989; Williams and Jarvis, 1991). However, the kinetic parameters of *cif* and *cit* NT processes resemble those of the *cib* process in as much that their apparent apparent K_m values are ≤ 40 μM .

The expression of a *cif* NT process, usually in conjunction with a second NT process, has been observed in the brush-border membranes of epithelial cells of intestine and kidney, choroid plexus, and in isolated splenocytes (Darnowski *et al.*, 1987), peritoneal macrophage (Baer and Moorji, 1990; Plagemann and Aran, 1990a; Plagemann and Aran, 1990b) and lymphocytes (Plagemann *et al.*, 1990; Plagemann and Aran, 1990b). In addition, the *cif* NT process has been observed in cultured cell lines of murine leukemia L1210 (Dagnino, 1988; Dagnino and Paterson, 1990; Dagnino *et al.*, 1991a), rat carcinosarcoma Walker 256 (Crawford and Belt, 1991), mouse macrophage RAW309 (Plagemann and Aran, 1990b), and mouse lymphoma S49 (Plagemann, 1991). The *cit* and *cib* NT processes have been observed in cultured pig kidney epithelial (Griffith *et al.*, 1992) and human carcinoma CaCo cells (Belt *et al.*, 1991), respectively. While the presence of concentrative NT processes appears not to be as widespread as the equilibrative NT processes, both equilibrative and concentrative processes have been observed to be simultaneously expressed in the majority of the cell types listed above.

Recently, an exception to the general rule of NBMPR-insensitivity of concentrative NT processes has been reported. Paterson and co-workers have identified a Na^+ -dependent NT process in freshly-isolated human chronic lymphocytic and acute myelogenous leukemia cells that is fully inhibited by low concentrations of NBMPR,

dipyridamole and dilazep (Paterson *et al.*, 1993). The permeant selectivity of this concentrative NBMPR-sensitive (*cs*) NT process is currently unknown.

Finally, a single case of a K⁺-dependent NT process in rat kidney brush-border membrane vesicles has been reported by Lee and co-workers (Lee *et al.* 1990; Jarvis *et al.*, 1989; Lee *et al.* 1990). The characterization of this putative K⁺-dependent NT process is very much incomplete and its existence has yet to be unequivocally demonstrated.

Identification of Proteins That Mediate Na⁺-Dependent Transport of Nucleosides

Functional expression of proteins that mediate the *cif*, *cit*, and *cib* NT processes has been accomplished by injection and expression of total cellular mRNA into *Xenopus laevis* oocytes (Jarvis and Griffith, 1991; Wu *et al.*, 1992; Huang *et al.*, 1993; Terasaki *et al.*, 1993). The technique of screening cDNA libraries by this means of functional expression in *X. laevis* oocytes has proved useful in the identification of cDNA that encode other concentrative transporters (For review see; Sigel, 1990). Recently, two different proteins that mediate Na⁺-dependent NT have been identified by functional expression of *in vitro* transcribed RNA (cRNA) in *X. laevis* oocytes.

Huang and co-workers have isolated a 2420 bp cDNA from rat intestine whose protein product, when expressed in *X. laevis* oocytes, mediates a Na⁺-dependent influx of uridine and thymidine (Huang *et al.*, 1994). Uridine and thymidine influx were mutually inhibited in the presence of each other, as well as in the presence of cytidine and adenosine, but not other purine nucleosides. This profile of permeant selectivity suggested the cDNA encoded a *cit* NT protein. As well, 2',3'-dideoxycytidine (ddC) and 3'-azido-3'-deoxythymidine (AZT) were permeants, albeit poor, of the *cit* transporter. NBMPR-insensitive uridine influx mediated by the recombinant protein exhibited an apparent K_m of 37 μM, a value in general agreement with K_m values observed in studies conducted with cells and membrane vesicles. The cDNA-encoded protein consisted of a 648 amino acid sequence that exhibited 14 hydrophobic potential membrane spanning regions and a predicted molecular weight of 71 kDa. Interestingly, regions of the rat *cit* NT protein exhibited homology with only one protein, the *nupC* H⁺/nucleoside transporter of *E. coli* (Munch-Petersen and Mygind, 1983).

A cDNA-encoded protein, termed SNST1 (sodium-nucleoside transporter 1), which mediates *cib*-type NT activity was identified on the basis of its similarity to the sodium-glucose transporter (SGLT) (Pajor and Wright, 1992). In *X. laevis* oocytes, SNST1-mediated uridine influx was inhibited by adenosine, cytidine and guanosine, a permeant selectivity exhibited by *cib*-type NT processes. Interestingly, 2',3'-dideoxycytidine (ddC) inhibited uridine uptake mediated by SNST1. This observation contradicts the permeant selectivity of the *cib* NT process as described from functional studies in rabbit choroid plexus (Wu *et al.*, 1992). The SNST1 protein is predicted to consist of a 672 amino acid sequence that contains 12 hydrophobic transmembrane regions. There is no similarity between the cloned rabbit SNST1 and the cloned rat *cif* NT protein described above. SNST1 is a member of the SGLT-like transporter family that includes members which mediate the flux of glucose, myoinositol, proline, and pantothenate (Wright *et al.*, 1992).

Regulated Expression of Nucleoside Transport During Cellular Proliferation and Differentiation

A correlation between NT activity and the proliferative status of cells was originally described in studies that examined serum- or insulin-induced uridine uptake in fibroblasts (Eilam and Bibi, 1977; Sander and Pardee, 1972; Stambrook and Siskin, 1972). A 2-fold increase in numbers of NBMPR binding sites, accompanied by 4- to 8-fold increases of V_{max} for nucleoside influx, was demonstrated to occur in synchronized HeLa cells upon entry into S phase of the cell cycle (Cass *et al.*, 1979). An S phase-related increase in NBMPR binding sites and NT activity was also observed in mouse S1 macrophage

cells that had been stimulated to proliferate with colony stimulating factor-1 (Meckling-Gill *et al.*, 1993). Similarly, increased numbers of NBMPR binding sites in freshly-isolated human lymphoma cells were shown to correlate with increases in proliferative rates (Wiley *et al.*, 1982; Wiley *et al.*, 1989). Thus, it appears that NT activity may be correlated with the proliferative status of mammalian cells.

Changes in the types and activities of equilibrative and concentrative NT processes have been linked to cellular differentiation in a number of different cell types. A striking example of this phenomenon is observed in human promyelocytic HL60 cells that have been induced along the myelocytic (Lee *et al.*, 1991) or granulocytic lineages (Chen *et al.*, 1986). Granulocytic maturation of HL60 cells was accompanied by decreases of 95% and 90% in numbers of NBMPR binding sites and rates of adenosine uptake, respectively. HL60 cells induced along the myelocytic pathway exhibited a 75% decrease in equilibrative NT activity paralleled by an increase of Na⁺-dependent NT activity. Maturation of sheep and guinea pig reticulocytes, which normally express high levels of *es* NT activity, into erythrocytes has been shown to be accompanied by a loss of NT activity and NBMPR binding sites (Jarvis and Young, 1982; Blostein and Grafova, 1987). A study of rat intestinal epithelial IEC-6 cells revealed that upon formation of confluent monolayer cultures, which is considered to be a differentiative event, a 3-fold increase of Na⁺-dependent NT activity was induced (Jacobs *et al.*, 1990). Expression of the Na⁺-dependent NT process was also found to be induced by the presence of hydroxycortisone, insulin or epidermal growth factor, all of which are considered to promote apical membrane functions and, hence, differentiation of IEC-6 cells.

These few examples presented suggest a role for regulated expression of NT activity in various cellular processes. Whether regulated expression is due to transcriptional or post-transcriptional control mechanisms is currently not known, although preliminary studies have uncovered cellular pathways involved in such changes in NT activity.

Cellular Mechanisms for Regulation of Nucleoside Transporter Activity

Two separate second messenger pathways have been demonstrated to alter the *in vivo* expression of the *es* NT process. Exposure of mouse S49 cells, which express primarily *es* NT, to ethanol leads to (i) activation of cAMP-dependent kinase and (ii) an "asymmetric" regulation of NT kinetics wherein influx is drastically reduced while efflux and numbers of NBMPR binding sites remain unchanged (Nagy *et al.*, 1990). The effect of ethanol on *es* NT activity could be mimicked by stimulation with forskolin, an activator of the cAMP-dependent kinase pathway, and was absent in mutant S49 cells that lacked the ability to be stimulated via the cAMP-dependent kinase pathway (Nagy *et al.*, 1991). Delicado and co-workers (1991) have shown that exposure of isolated bovine chromaffin cells to agents that activate protein kinase C results in an immediate decrease (~50%) in NT activity and numbers of NBMPR binding sites.

Together, these studies indicate that a phosphorylation event mediated by either cAMP-dependent kinase or protein kinase C directly alters the functional and/or structural nature of the *es* NT process.

OBJECTIVES OF THE THESIS

The functional characteristics of mammalian NT processes have been the subject of intense study during the past twenty years, yet until recently little was known of the structure of NT proteins or the genes that encode them. The material presented in the chapters of this thesis represent the results of individual studies undertaken to identify the structural characteristics important for the function of these NT proteins.

Specifically, the objectives of this research project were to:

- (i) determine whether the presence of N-linked oligosaccharide influences the function and/or expression of NT proteins.
- (ii) identify eucaryotic cell types that may be useful for identification of recombinant equilibrative NT proteins in a functional expression cloning procedure.
- (iii) develop a functional expression cloning methodology for use in the identification of cDNA-encoded proteins that mediate equilibrative NT.
- (iv) characterize recombinant NT proteins that had been identified by a functional expression cloning method.

BIBLIOGRAPHY

- Agbanyo, F. R., Cass, C. E., and Paterson, A. R. P. (1988) External location of sites on pig erythrocyte membranes that bind nitrobenzylthioinosine. *Molec. Pharmacol.* **33**, 332-337.
- Aronow, B., Allen, K., Patrick, J., and Ullman, B. (1985) Altered nucleoside transporters in mammalian cells selected for resistance to physiological effects of inhibitors of nucleoside transport. *J. Biol. Chem.* **260**, 6226-6233.
- August, M. E., Birks, E. M., and Prusoff, W. H. (1991) 3'-Deoxythymidin-2'-ene permeation of human lymphocyte H9 cells by nonfacilitated diffusion. *Mol. Pharmacol.* **39**, 246-249.
- Belt J. A., Phelps, D. A., and Crawford, C. R. (1991) Na⁺-dependent, concentrative nucleoside transport in human colon carcinoma cells. *Proc. Amer. Assoc. Cancer Res.* **32**, 16.
- Belt, J. A. and Noel, D. L. (1985) Nucleoside transport in Walker 256 rat carcinosarcoma and S49 mouse lymphoma cells. *Biochem. J.* **232**, 681-688.
- Belt, J. A. and Noel, D. L. (1988) Isolation and characterization of a mutant of L1210 murine leukemia deficient in nitrobenzylthioinosine-insensitive nucleoside transport. *J. Biol. Chem.* **263**, 13879-13822.
- Blostein, R. and Grafova, E. (1987) Characteristics of membrane transport losses during reticulocyte maturation. *Biochem. Cell Biol.* **65**, 869-875.
- Boleti, B. (1991) Nucleoside transport in K562 leukemia cells. Ph.D. thesis, Department of Biochemistry, University of Alberta, Edmonton, Alberta.
- Boumah, C. E., Hogue, D. L., and Cass, C. E. (1992) Expression of high levels of nitrobenzylthioinosine-sensitive nucleoside transport in cultured human choriocarcinoma (BeWo) cells. *Biochem. J.* **288**, 987-996.
- Brett, C. M., Washington, C. B., Ott, R. J., Gutierrez, M. M. and Giacomini, K. M. (1993) Interaction of nucleoside analogues with the sodium-nucleoside transport system in brush border membrane vesicles from human kidney. *Pharm. Res.* **10**, 423-426.
- Cabantchic, Z. I. and Ginsburg, H. (1977) Transport of uridine in human red blood cells. Demonstration of a simple carrier-mediated process. *J. Gen. Physiol.* **69**, 75-96.
- Cass, C. E. and Paterson, A. R. P. (1972) Mediated transport of nucleosides in human erythrocytes. Accelerative exchange diffusion of uridine and specificity towards pyrimidine nucleosides as permeants. *J. Biol. Chem.* **247**, 3314-3320.
- Cass, C. E., Belt, J. A., and Paterson, A. R. P. (1987) Adenosine transport in cultured cells and erythrocytes. *Prog. Clin. Biol. Res.* **230**, 13-40.
- Cass, C. E., Dahlig, E., Lau, E. Y., Lynch, T. P., and Paterson, A. R. P. (1978) Fluctuations in nucleoside uptake and binding of the inhibitor of nucleoside transport, nitrobenzylthioinosine, during the replication cycle of HeLa cells. *Cancer Res.* **39**, 1245-1252.

- Cass, C. E., Gaudette, L. A., and Paterson, A. R. P. (1974) Mediated transport of nucleoside in human erythrocytes: specific binding of the inhibitor nitrobenzylthioinosine to nucleoside transport sites in the erythrocyte membrane. *Biochim. Biophys. Acta.* **345**, 1-10.
- Cass, C. E., Kolassa, N., Uehara, Y., Dahling - Harley, E., Harley, E. R., and Paterson, A. R. P. (1981) Absence of binding sites for the transport inhibitor nitrobenzylthioinosine on nucleoside transporter-deficient mouse lymphoma cells. *Biochim. Biophys. Acta.* **649**, 769-777.
- Che, M., Nishida, T., Gatmaitan, Z., and Arias, I. M. (1992) A nucleoside transporter is functionally linked to ectonucleotidases in the rat liver canalicular membrane. *J. Biol. Chem.* **267**, 9684-9688.
- Chen, S. F., Cleaveland, J. S., Hollman, A. B., Wiemann, M. C., Parks, R. E. Jr., and Stoeckler, J. D. (1986) Changes in nucleoside transport of HL-60 human promyelocytic cells during N,N-dimethylformamide induced differentiation. *Cancer Res.* **46**, 3449-3455.
- Crawford, C. R., Ng, C. Y. C., and Belt, J. A. (1990b) Isolation and characterization of a L1210 cell line retaining the sodium-dependent carrier *cif* as its sole nucleoside transport activity. *J. Biol. Chem.* **265**, 13730-13734.
- Crawford, C. R., Ng, C. Y. C., Ullman, B., and Belt, J. A. (1990a) Identification and reconstitution of the nucleoside transporter of CEM human leukemia cells. *Biochim. Biophys. Acta.* **1024**, 289-297.
- Dagnino, L (1988) Sodium-driven nucleoside transport in mouse leukemia L1210 cells. Ph.D. thesis, Department of Pharmacology, University of Alberta, Edmonton, Alberta.
- Dagnino, L. and Paterson, A. R. P. (1990) Sodium-dependent and equilibrative nucleoside transport systems in L1210 mouse leukemia cells: effect of inhibitors of equilibrative systems on the content and retention of nucleosides. *Can. Res.* **50**, 6549-6553.
- Dagnino, L., Bennet Jr., L.L., and Paterson, A. R. P. (1991a) Sodium-dependent nucleoside transport in mouse leukemia L1210 cells. *J. Biol. Chem.* **266**, 6308-6311.
- Dagnino, L., Bennet Jr., L.L., and Paterson, A. R. P. (1991b) Substrate specificity, kinetics, and stoichiometry of sodium-dependent adenosine transport in L1210/AM mouse leukemia cells. *J. Biol. Chem.* **266**, 6312-6317.
- Darnowski, J. W., Holdridge, C., and Handschumacher, R. E. (1987) Concentrative uridine transport by murine splenocytes: kinetics, substrate specificity, and sodium dependency. *Cancer Res.* **47**, 2614-2619.
- Delicado, E. G., Sen, R. P., and Miras-Portugal, T. M. (1991) Effects of phorbol esters and secretagogues on nitrobenzylthioinosine binding to nucleoside transporters and nucleoside transport in cultured chromaffin cells. *Biochem. J.* **279**, 651-655.
- Domin, B. A., Mahoney, W. B., and Zimmerman, T. P. (1988) 2',3'-Dideoxythymidine permeation of human erythrocyte membrane by nonfacilitated diffusion. *Biochem. Biophys. Res. Commun.* **154**, 825-831.

Eilam, Y. and Bibi, O. (1977) Distinct properties of uridine transport systems in growing, quiescent and serum-stimulated hamster embryo cells. *Biochim. Biophys. Acta.* **467**, 51-64.

Fleming, S. A., Rawling, D. B., and Robins, M. J. (1990) Photochemistry of the nucleoside membrane transport inhibitor 6[(4-nitrobenzyl)thio]-9-(β -D-ribofuranosyl)-purine. *Tetrahedron Letters* **31**, 4995-4998.

Gati, W. P. and Paterson, A. R. P. (1989) Interaction of [^{3} H]dilazep at nucleoside transporter-associated binding sites on S49 mouse lymphoma cells. *Mol. Pharmacol.* **36**, 134-141.

Gati, W. P. and Paterson, A. R. P. (1989) Nucleoside transport. In: *Red Blood Cell Membranes; Structure, Function and Clinical Implications*. (Agre, P. and Parker, J. C., eds.) Marcel Dekker, New York.

Gati, W. P., Belt, J. A., Jacobs, E. S., Young, J. D., and Paterson, A. R. P. (1986) Photoaffinity labeling of a nitrobenzylthioinosine-binding polypeptide from cultured Novikoff hepatoma cells. *Biochem. J.* **236**, 665-670.

Gati, W. P., Dagnino, L., and Paterson, A. R. P. (1989) Enantiomeric selectivity of adenosine transport systems in mouse erythrocytes and L1210 cells. *Biochem. J.* **263**, 957-960.

Gati, W. P., Misra, H. K., Knaus, E. E., and Wibe, L. I. (1984) Structural modifications at the 2'- and 3'-positions of some pyrimidine nucleosides as determinants of their interaction with the mouse erythrocyte nucleoside transporter. *Biochem. Pharmacol.* **33**, 3325-3331.

Gati, W. P., Paterson, A. R. P., Tyrell, D. L. J., Cass, C. E., Moravek, J., and Robbins, M. J. (1992) Nucleobase transporter-mediated permeation of 2',3'-dideoxyguanosine in human erythrocytes and human T-lymphoblastoid CCRF-CEM cells. *J. Biol. Chem.* **267**, 22272-22276.

Good, H. A., Craik, J. D., Jarvis, S. M., Kwong, F. Y. P., Young, J. D., Paterson, A. R. P., and Cass, C. E. (1987) Characterization of monoclonal antibodies that recognize band 4.5 polypeptides associated with nucleoside transport in pig erythrocytes. *Biochem. J.* **244**, 749-755.

Griffiths, D. A., Doherty, A. J., and Jarvis, S. M. (1992) Nucleoside transport in cultured LLC-PK1 epithelia. *Biochim. Biophys. Acta.* **1106**, 303-310.

Gutierrez, M. M. and Giacomini, K. M. (1993) Substrate selectivity, potential sensitivity and stoichiometry of Na⁺-nucleoside transport in brush border membrane vesicles from human kidney. *Biochim. Biophys. Acta.* **1149**, 202-208.

Gutierrez, M. M., Brett, C. M., Ott, R. J., Hui, A. C., and Giacomini, K. M. (1992) Nucleoside transport in brush border membrane vesicles from human kidney. *Biochim. Biophys. Acta.* **1105**, 1-9.

Hammond, J. R. and Johnstone, R. M. (1989) Solubilization and reconstitution of a nucleoside-transport system from Ehrlich ascites-tumour cells. *Biochem. J.* **262**, 109-118

Hogue, D. L., Hodgson, K. C., and Cass, C. E. (1990) Effects of inhibition of N-linked glycosylation by tunicamycin on nucleoside transport polypeptides of L1210 leukemia cells. *Biochem. Cell Biol.* **68**, 199-209.

Huang, Q. Q., Harvey, C. M., Paterson, A. R. P., Cass, C. E., and Young J. D. (1993) Functional expression of Na⁺-dependent nucleoside transport systems of rat intestine in isolated oocytes of *Xenopus laevis*. *J. Biol. Chem.* **268**, 20613-20619.

Huang, Q. Q., Yao, S. Y. M., Ritzel, M. W. L., Paterson, A. R. P., Cass, C. E., and Young J. D. (1994) Cloning and functional expression of a complementary DNA encoding a mammalian nucleoside transporter protein. Submitted for Publication.

Ijzerman, A. P., Menkveld, G. J., and Thedinga, K. H. (1989) A refined method for the photoaffinity labelling of the nitrobenzylthioinosine-sensitive nucleoside transport protein: application to cell membranes of calf lung. *Biochim. Biophys. Acta.* **979**, 153-156.

Jacobs, E. S., Os-Corby, D. J., and Paterson, A. R. P. (1990) Expression of sodium-linked nucleoside transport activity in monolayer cultures of IEC-6 intestinal epithelial cells. *J. Biol. Chem.* **265**, 22210-22216.

Jarvis, S. M. (1986) Nitrobenzylthioinosine-sensitive nucleoside transport system: mechanism of inhibition by dipyrindamole. *Mol. Pharmacol.* **30**, 659-665.

Jarvis, S. M. and Griffith, D. A. (1991) Expression of the rabbit intestinal N² Na⁺/nucleoside transporter in *Xenopus laevis* oocytes. *Biochem. J.* **278**, 605-607.

Jarvis, S. M. and Young, J. D. (1982) Nucleoside translocation in sheep reticulocytes and fetal erythrocytes: a proposed model for the nucleoside transporter. *J. Physiol.* **324**, 47-66.

Jarvis, S. M. and Young, J. D. (1986) Nucleoside transport in rat erythrocytes: two components with differences in sensitivity to inhibition by nitrobenzylthioinosine and *p*-chloromercuriphenyl sulfonate. *J. Membrane Biol.* **93**, 1-10.

Jarvis, S. M. and Young, J. D. (1987) Photoaffinity labelling of nucleoside transporter polypeptides. *Pharmac. Ther.* **32**, 339-359.

Jarvis, S. M., Hammond, J. R., Paterson, A. R. P., and Clanachan, A. S. C. (1982) A study of uridine transport and nitrobenzylthioinosine binding by mammalian erythrocytes. *Biochem. J.* **208**, 2202-2208.

Jarvis, S. M., Hammond, J. R., Paterson, A. R. P., and Clanachan, A. S. C. (1983a) Nucleoside transport in human erythrocytes. A simple carrier with directional symmetry in fresh cells but with directional asymmetry in cells from outdated blood. *Biochem J.* **210**, 457-461.

Jarvis, S. M., Janmohamed, S. N., and Young, J. D. (1983b) Kinetics of nitrobenzylthioinosine binding to the human erythrocyte transporter. *Biochem. J.* **216**, 661-667.

- Jarvis, S. M., Williams, T. C., Lee, C., and Cheeseman, C. I. (1989) Active transport of nucleosides and nucleoside drugs. *Biochem. Soc. Trans.* **17**, 448-450.
- Jones, K. W. and Hammond, J. R. (1992) Heterogeneity of [^3H]dipyridamole binding to the CNS membrane: correlation with [^3H]nitrobenzylthioinosine binding and [^3H]uridine influx studies. *J. Neurochem.* **59**, 1363-1371.
- Kwong, F. Y. P., Baldwin, S. A., Scudder, P. R., Jarvis, S. M., Choy, M. Y. M., and Young, J. D. (1986) Erythrocyte nucleoside and sugar transport. *Biochem. J.* **240**, 349-356.
- Kwong, F. Y. P., Davies, A., Tse, M. C., Young, J. D., Henderson, P. J. F., and Baldwin, S. A. (1988) Purification of the human erythrocyte nucleoside transporter by immunoaffinity chromatography. *Biochem. J.* **255**, 243-249.
- Kwong, F. Y. P., Fincham, H. E., Davies, A., Beaumont, N., Henderson, P. J. F., Young, J. D., and Baldwin, S. A. (1992) Mammalian nitrobenzylthioinosine-sensitive nucleoside transport proteins. *J. Biol. Chem.* **267**, 21954-21960.
- Kwong, F. Y. P., Wu, R. J. S., Shi, M. M., Fincham, H. E., Davies, A., Henderson, P. J. F., Baldwin, S. A., and Young, J. D. (1993) Enzymatic cleavage as a probe of the molecular structures of mammalian equilibrative nucleoside transporters. *J. Biol. Chem.* **268**, 22127-22134.
- Lee, C. W., Cheeseman, C. I., and Jarvis, S. M. (1988) Na^+ - and K^+ -dependent uridine transport in rat renal brush-border membrane vesicles. *Biochim. Biophys. Acta.* **942**, 139-149.
- Lee, C. W., Solokowski, J. A., Sartotelli, A. C., and Handschumacher, R. E. (1991) Induction of the differentiation of HL-60 cells by phorbol 12-myristate 13-acetate activates a Na^+ -dependent uridine-transport system. *Biochem. J.* **274**, 85-90.
- Lee, C., W. Cheeseman, C. I., and Jarvis, S. M. (1990) Transport characteristics of the renal brush border Na^+ - and K^+ -dependent uridine carriers. *Amer. J. Physiol.* **258**, F1203-F1210.
- Meckling-Gill, K. A., Guilbert, L. and Cass, C. E. (1993) CSF-1 stimulates nucleoside transport in S1 macrophages. *J. Cell. Physiol.* **155**, 530-538.
- Munch-Petersen, A. and Mygind, B. (1983) In: *Metabolism of Nucleotides, Nucleosides and Nucleobases in Microorganisms* (Munch-Petersen, A., ed.) Academic Press, London.
- Murray, A. W. (1971) The biological significance of purine salvage. *Ann. Rev. Biochem.* **40**, 811-826.
- Nagy, L. E., Diamond, I., and Gordon, A. S. (1991) cAMP-dependent protein kinase regulates inhibition of adenosine transport by ethanol. *Mol. Pharmacol.* **40**, 812-817.
- Nagy, L. E., Diamond, I., Casso, D. J., Franklin, C., and Gordon, A. S. (1990) Ethanol increases extracellular adenosine by inhibiting adenosine uptake via the nucleoside transporter. *J. Biol. Chem.* **265**, 1946-1951.

- O'Conner, S. E., Dainty, J. A., and Leff, P. (1991) Further classification of ATP receptors based on agonist studies. *Trends Pharmacol. Sci.* **12**, 137-141.
- Pajor, A. M. and Wright, E. M. (1992) Cloning and expression of a mammalian Na^+ /nucleoside cotransporter. *J. Biol. Chem.* **267**, 3557-3560.
- Paterson, A. R. P., Clanahan, A. S., Craik, J. D., Gati, W. P., Jakobs, E. S., Wiley, J. S., and Cass, C. E. (1991) Plasma membrane transport of nucleosides, nucleobases, and nucleotides: an overview. In: Role of Adenosine and Adenine Nucleotides in the Biological System. (Imai, S. and Nakazawa, M., eds) Elsevier, Tokyo.
- Paterson, A. R. P., Gati, W. P., Vijayalakshmi, D., Cass, C. E., Mant, M. J., Young, J. D., and Belch, A. R. (1993) Inhibitor-sensitive, Na^+ -linked transport of nucleoside transport analogs in leukemia cells from patients. *Proc. Amer. Assoc. Cancer Res.* **34**, 14.
- Paterson, A. R. P., Jakobs, E. S., Harley, E. R., Fu, N. W., Robins, M. J., and Cass, C. E. (1983) Inhibition of nucleoside transport. In: Regulatory Function of Adenosine. (Berne, R. M., Rall, T. W., and Rubio, R., eds.) Martinus Nijhoff, The Hague.
- Paterson, A. R. P., Lau, E. Y., Dahlig, E., and Cass, C. E. (1980) A common basis for inhibition of nucleoside transport by dipyrindamole and nitrobenzylthioinosine? *Mol. Pharmacol.* **18**, 40-44.
- Paul, B., Chen, M. F., and Paterson, A. R. P. (1975) Inhibitors of nucleoside transport. A structure-activity study using human erythrocytes. *J. Med. Chem.* **18**, 968-973.
- Plagemann, P. G. W. and Woffendin, C. (1989a) Permeation and salvage of dideoxyadenosine in mammalian cells. *Mol. Pharmacol.* **36**, 185-192.
- Plagemann, P. G. W. and Woffendin, C. (1989b) Dideoxycytidine permeation and salvage by mouse leukemia cells and human erythrocytes. *Biochem. Pharmacol.* **38**, 3469-3475.
- Plagemann, P. G. W. and Aran, J. P. (1990a) Na^+ -dependent, active nucleoside transport in mouse spleen lymphocytes, leukemia cells, fibroblasts and macrophages, but not in equivalent human or pig cells; dipyrindamole enhances nucleoside salvage by cells with both active and facilitated transport. *Biochim. Biophys. Acta.* **1025**, 32-42.
- Plagemann, P. G. W. and Aran, J. P. (1990b) Characterization of Na^+ -dependent, active nucleoside transport in rat and mouse peritoneal macrophages, a mouse macrophage cell line and normal rat kidney cells. *Biochim. Biophys. Acta.* **1028**, 289-298.
- Plagemann, P. G. W. and Wohlheuter, R. M. (1984) Effect of temperature on kinetics and differential mobility of empty and loaded nucleoside transporter of human erythrocytes. *J. Biol. Chem.* **259**, 9024-9027.
- Plagemann, P. G. W. and Wohlheuter, R. M. (1985) Nitrobenzylthioinosine-sensitive and resistant nucleoside transport in normal and transformed cells. *Biochim. Biophys. Acta.* **816**, 387-395.
- Plagemann, P. G. W., Aran, J. P., and Woffendin, C. (1990) Na^+ -dependent, active and Na^+ -independent, facilitated transport of formycin B in mouse spleen lymphocytes. *Biochim. Biophys. Acta.* **1022**, 93-102.

- Plagemann, P. G. W., Wohlheuter, R. M., and Woffendin, C. (1988) Nucleoside and nucleobase transport in animal cells. *Biochim. Biophys. Acta.* **947**, 405-433.
- Ramkumar, V., Pierson, G., and Stiles, G. (1988) Adenosine receptors: clinical implications and biochemical mechanisms. *Prog. Drug Res.* **32**, 195-247.
- Rudolphi, K. A., Schubert, P., Parkinson, F. E., and Fredholm, B. B. (1992) Adenosine and brain ischemia. *Cerebrovas. Brain Metab. Rev.* **4**, 346-369.
- Sander, G. and Pardee, A. B. (1972) Transport changes in synchronously growing CHO and L cells. *J. Cell Physiol.* **80**, 267-272.
- Shi, M. M. and Young, J. D. (1986) [³H]Dipyridamole binding to nucleoside transporters from guinea-pig and rat lung. *Biochem. J.* **240**, 879-883.
- Sigel, E. (1990) Use of *Xenopus* oocytes for the functional expression of plasma membrane proteins. *J. Memb. Biol.* **117**, 201-221.
- Stambrook, P. J. and Siskin, J. E. (1972) Induced changes in the rates of uridine-³H uptake and incorporation during the G₁ and S periods of synchronized Chinese hamster cells. *J. Cell Biol.* **52**, 514-525.
- Stiles, G. L. (1992) Adenosine receptors. *J. Biol. Chem.* **267**, 6451-6454.
- Terasaki, T., Kadowaki, A., Higashida, H., Nakayama, K., Tamai, I., and Tsuji, A. (1993) Expression of the Na⁺-dependent nucleoside transport system in rabbit small intestine: studies with MRNA-injected *Xenopus laevis* oocytes. *Biol. Pharm. Bull.* **16**, 493-496.
- Vijayalakshmi, D., Dagnino, L., Belt, J. A., Gati, W. P., Cass, C. E., and Paterson, A.R. P. (1992) L1210/B23.1 cells express equilibrative, inhibitor-sensitive nucleoside transport activity and lack two parental nucleoside transport activities. *J. Biol. Chem.* **267**, 16951-16956.
- Vijayalakshmi, D. and Belt, J. A. (1988) Sodium-dependent nucleoside transport in mouse intestinal epithelial cells. *J. Biol. Chem.* **263**, 19419-19423.
- Vijayalakshmi, D., Robbins, M. J., Belt, J. A., Gati, W. P., Cass, C. E., and Paterson, A.R. P. (1994a) Interaction of ribofuranosyl-modified analogs of adenosine with the NBMPR-sensitive nucleoside transporter. *In Preparation*.
- Vijayalakshmi, D., Robbins, M. J., Belt, J. A., Gati, W. P., Cass, C. E., and Paterson, A.R. P. (1994b) Structural determinants of adenosine permeation via the NBMPR-insensitive nucleoside transporter. *In Preparation*.
- Wiley, J. S., Snook, M. B., and Jamieson, G. P. (1989) Nucleoside transport in acute leukemia and lymphoma: close relation to proliferative rate. *Brit. J. Haematol.* **71**, 203-207.
- Wiley, J. S., Taupin, J., Jamieson, G. P., Snook, M. B., Sawyer, W. H., and Finch, L. R. (1982) Cytosine arabinoside transport and metabolism in acute leukemias and T cell lymphoblastic lymphoma. *J. Clin. Invest.* **75**, 632-642.

- Williams, T. C. and Jarvis, S. M. (1991) Multiple sodium-dependent nucleoside transporter systems in bovine renal brush-border vesicles. *Biochem. J.* **274**, 27-33.
- Woffendin, C. and Plagemann, P. G. W. (1987) Interaction of [³H]dipyridamole with the nucleoside transporters of human erythrocytes and cultured animal cells. *J. Membr. Biol.* **98**, 89-100.
- Wohlheuter, R. M. and Plagemann, P. G. W. (1982) On the functional symmetry of nucleoside transport in mammalian cells. *Biochim. Biophys. Acta.* **689**, 249-260.
- Wright, E. M., Hager, K. M., and Turk, E. (1992) Sodium cotransport proteins. *Curr. Opin. Cell Biol.* **4**, 696-702.
- Wu, X., Yuan, G., Brett, C. M., Hui, A. C., and Giacomini, K. M. (1992) Sodium-dependent nucleoside transport in choroid plexus from rabbit. *J. Biol. Chem.* **267**, 8813-8818.
- Young, J. D., Jarvis, S. M., Belt, J. A., Gati, W. P., and Paterson, A. R. P. (1984) Identification of the nucleoside transporter in cultured mouse lymphoma cells. *J. Biol. Chem.* **259**, 8363-8365.
- Young, J. D., Jarvis, S. M., Robins, M. J., and Paterson, A. R. P. (1983) Photoaffinity labeling of the human erythrocyte nucleoside transporter by N⁶-(p-aminobenzyl)adenosine and nitrobenzylthioinosine. *J. Biol. Chem.* **258**, 2202-2208.
- Zimmerman, T. P., Mahony, W. B., and Prus, K. L. (1987) 3'-Azido-3'-deoxythymidine. *J. Biol. Chem.* **262**, 5748-5754.
- Zimmermann, H. (1992) 5'-Nucleotidase: molecular structure and functional aspects. *Biochem. J.* **285**, 345-365.

Chapter II

A ROLE FOR N - LINKED OLIGOSACCHARIDE IN THE FUNCTIONAL EXPRESSION OF NUCLEOSIDE TRANSPORTERS OF L1210/C2 CELLS¹

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INTRODUCTION

The ability to covalently link chains of oligosaccharide to asparagine (N) residues of proteins is shared by almost all eucaryotic cells (Hart, 1992). The biosynthesis of the core structure of the N-linked oligosaccharide chain occurs in the endoplasmic reticulum (ER) by a common pathway (Kornberg and Kornberg, 1985; Abeijon and Hirschberg, 1992). The core oligosaccharide is synthesized by sequential transferase-mediated additions of N-acetylglucosamine, mannose, and glucose residues onto a phosphate-dolichol lipid embedded in the ER membrane (Fig. II 1). The core oligosaccharide is subsequently covalently attached, as a co-translational process in the lumen of the ER, via a N-acetylglucosaminylasparagine linkage to asparagine residues present in the consensus amino acid sequence Asn-X-Ser/Thr. The selection of a N-linked glycosylation site(s) within a given protein has been observed to be both species- and tissue-specific (Rademacher *et al.*, 1988). This heterogeneity of site selection can lead to the generation of protein glycoforms, as does the further processing of oligosaccharide chains within the Golgi compartment.

The processing of the core oligosaccharide chain in the Golgi compartment can generate oligosaccharide chains of either of three basic types; high-mannose, complex, or hybrid types (Fig. II 2). While all N-linked glycoproteins share a common pentasaccharide core oligosaccharide structure ($\text{Man}\alpha 1-3(\text{Man}\alpha 1-6)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc-Asn}$), single types of protein have been demonstrated to simultaneously display different types of oligosaccharide chains and/or the same type of oligosaccharide chains with variable processing (Rademacher *et al.*, 1988). High-mannose type structures contain two to six mannose residues attached to the pentasaccharide core. Complex type structures contain N-acetylglucosamine linked to the terminal mannose residues of the pentasaccharide core and may contain two (biantennary), three (triantennary), or four (tetraantennary) branching N-acetylglucosamine residues. These structures may also exhibit a bisecting N-acetylglucosamine linked $\beta 1-4$ to the β -linked mannose of the pentasaccharide core. Hybrid type structures exhibit the bisecting N-acetylglucosamine linked $\beta 1-4$ to the β -linked mannose of the pentasaccharide core and both mannose and N-acetylglucosamine residues attached to the terminal mannose residues. Increased levels of oligosaccharide complexity attained by the differential addition of sugars (such as fucose and sialic acid) also contribute to the generation of large pools of protein glycoforms.

The role of N-linked glycosylation as a determinant of biological activity has been demonstrated for many different proteins (for review, see Rademacher *et al.*, 1988). Since this study investigated the potential role of N-linked glycosylation on the function of plasma membrane NT proteins, previous studies of glycosylation of integral plasma membrane proteins are of particular interest. Many of these studies have utilized tunicamycin, a specific inhibitor of the transferase responsible for the addition of N-acetylglucosamine to dolichol-phosphate in the first step of core N-linked oligosaccharide biosynthesis (see Fig. II 1), to examine the role of N-linked glycosylation. The structure of tunicamycin, a nucleoside antibiotic that is usually isolated as a functionally identical mixture of species, is shown in Fig. II 3. Tunicamycin consists of uracil, linked in a N-glycosidic bond to tunicamine, an 11-carbon aminodeoxydialdose (for review; see Elbein, 1987). Inhibition of N-linked glycosylation by tunicamycin has implicated N-linked glycosylation in cell-surface expression of a number of integral membrane glycoproteins, including the acetylcholine (Prives and Bar-Sagi, 1983), IgE (Keegan and Conrad, 1987), and transferrin receptors (Hunt *et al.*, 1989) and CD45 (Pulido and Sanchez-Madrid, 1992). Kinetic parameters for receptor-ligand or transporter-permeant interaction have been demonstrated to be N-linked glycosylation-dependent for the transferrin receptor

(Hunt *et al.*, 1989), Na⁺/K⁺ antiporter (Alboim *et al.*, 1992), organic cation transporter (Ott *et al.*, 1992), Na⁺/H⁺ antiporter (Yusufi *et al.*, 1988) and HepG2-type glucose transporter (Maher and Harrison, 1991). Interestingly, N-linked glycosylation of the insulin receptor β subunit (Leconte *et al.*, 1992) and β -adrenergic receptor (Boege *et al.*, 1988) is essential for signal transduction. Thus, it is apparent that N-linked glycosylation is required in many instances for proper functional expression of integral membrane proteins.

The *es* NT protein of erythrocytes from several species (Kwong *et al.*, 1993; Kwong *et al.*, 1992; Kwong *et al.*, 1986; Jarvis and Young, 1987; Good *et al.*, 1987), of rat and guinea pig liver (Kwong *et al.*, 1993; Jarvis and Young, 1987), and human K562 erythroleukemia cells (Boleti, 1991) have been shown to contain N-linked oligosaccharide, however the role of this N-linked glycosylation in the function of the *es* NT process has not yet been determined. This study utilized tunicamycin to examine whether N-linked oligosaccharide plays a role in the functional expression of the *es*, *ei*, and *cif* NT processes of L1210/C2 cells. In addition, we have examined the extent and type of N-linked oligosaccharide present on the *es* NT protein by endoglycosidase and chemical hydrolysis studies.

MATERIALS AND METHODS

Materials

Fisher's medium, RPMI 1640 medium, horse serum, fetal bovine serum, and gentamycin were obtained from Gibco Laboratories; L-[4,5-³H]leucine, [¹⁴C(U)]sucrose, [³H]H₂O, [1,2-¹⁴C]polyethylene glycol, Freon-TF (1,1,2-trichloro-trifluoroethane), Protosol, and Econofluor were from DuPont Canada Inc.; D-[5,6-³H]uridine, D-[methyl-³H]thymidine and D-[2-³H]mannose were from ICN Biomedicals Inc.; light paraffin oil (Saybolt Viscosity 125-135) was from Fisher Scientific; silicon 550 oil was from Dow Corning Corp.; dilazep hydrochloride was a gift from F. Hoffman-LaRoche; [³H]NBMPR was from Moravsek Biochemicals; tunicamycin, phenylmethylsulfonyl fluoride (PMSF), trifluoromethanesulfonic acid (TFMS), and protein molecular weight standards (Dalton Mark VII-L) were from Sigma Chemical Co.; endo- α -N-acetyl-galactosaminidase (O-glycanase) and peptide-N⁴[N-acetyl- β -glucosaminyl]asparagine amidase (N-glycanase) were from Genzyme Corp.; neuraminidase, leupeptin, aprotinin, and endo- β -N-acetylglucosaminidase F (endoglycosidase F) were from Boehringer Mannheim; Percoll was from Pharmacia Chemicals; tricaprlyl tertiary amine (Alamine 336) was from Henkel Co.; cellulose thin layer chromatography (TLC) sheets (Type 5577) were from Merck; and protein molecular weight standards were from Sigma Chemical Co. and Pharmacia Chemicals.

Cells and Culture Conditions

The murine leukemia cell line L1210/C2 was cloned from *in vivo*-passaged leukemia L1210 (Cass and Au-Yeung, 1976) that originated from a DBA/2 mouse (Dunham and Stewart, 1953). L1210/C2 cells were maintained as suspension cultures in either Fisher's medium or RPMI 1640 supplemented with NaHCO₃ (2 g/l) and 10% horse serum in the absence of antibiotics. Cultures were maintained at 37°C, in 95% air and 5% CO₂ with 95% humidity. Cell concentrations of cultures were determined using a Coulter Counter Model ZF electronic particle counter (Coulter Electronics Inc.) and stock cultures were diluted to 0.1 x 10⁵ - 0.2 x 10⁵ cells/ml every 2-3 days. Cell cultures were maintained

for a maximum of 30-40 generations. Large cultures of suspended cell cultures were generated in 1330-cm² glass roller bottles (50 rpm, Bellco) in medium that usually contained gentamycin (50 µg/ml). Experiments in this study utilized cell cultures that exhibited exponential cell proliferation (density $\leq 7 \times 10^5$ cells/ml).

Cell Proliferation Studies

The effect of tunicamycin on cell proliferation rates was determined using an established method (Cass *et. al.*, 1979). Cells were initially seeded, in triplicate, at 1×10^5 cells/ml in bottles containing fresh growth medium that lacked antibiotic. Graded concentrations of the drug were added to the bottles and cell concentrations were measured at 24-hr intervals thereafter. The effect of a concentration of drug on cell proliferation was ascertained by comparison of cell growth in the absence of drug to that in the presence of drug. The concentration of drug that inhibited the rate of cell proliferation by 50% (IC₅₀) was calculated for each time period.

Measurement of Precursor Incorporation

Cells were grown in media that contained 0.25 µCi L-[³H]leucine/ml, 0.25 µCi D-[³H]thymidine/ml, or 1.25 µCi D-[³H]mannose/ml and precursor incorporation was terminated by addition of equal volumes of ice-cold growth medium followed by centrifugation (150 x g, 10 min, 4°C). Cell pellets were suspended in 500 µl of 1% (w/v) phosphotungstic acid in 0.5 N HCl and centrifuged (9000 x g, 2 min). The pellets were washed twice in 10% (w/v) trichloroacetic acid and extracted with 500 µl of 95% ethanol/anhydrous ether (50:50, v/v). The acid-insoluble precipitates were collected by centrifugation (9000 x g, 2 min), solubilized with 250 µl of 0.5 N KOH, and neutralized with 200 µl of 0.5 N HCl. The protein content of this material was measured by the method of Bradford (Bradford, 1976), and the radioactivity of samples was determined by the addition of 5 ml xylene-detergent scintillant (Pande, 1976) followed by liquid scintillation counting.

Measurement of Nucleoside Influx in Cultured Cells

a) Buffers and Solutions Used in Transport Assays

i. Transport Oil

The oil mixture utilized in transport, binding, and metabolism assays was prepared by mixing approximately 75 ml paraffin oil (Saybolt Viscosity 125-135) with 425 ml silicon 550 oil. The final specific gravity (Specific Gravity/Baume Hydrometer) was adjusted to 1.032 g/ml using either type of oil.

ii. Nucleoside, Nucleobase, and NT Inhibitor Solutions

Sterile stock solutions of non-radioactive nucleosides and NT inhibitors were prepared by dissolving the compound in sterile dH₂O and filtering through a sterile 0.2 µm Millex-GV filter (Millipore). All solutions were stored at -20°C. Concentration of stock solutions was determined by UV absorbance using the molar extinction coefficient ($\bar{\epsilon}$) of the compounds at a wavelength of maximum absorption ($\lambda_{\max}$), as listed below. The concentration of working solutions, created by dilution of stock solutions, was ascertained by UV absorbance prior to use. Radiolabeled nucleosides were purified with a Varian 5000 high-pressure liquid chromatography instrument using water-methanol gradients on a C18 reverse phase column, as described in detail elsewhere (Boleti, 1991). Purified nucleosides were stored as water-methanol solutions at 4°C and were used within 2 months of the purification date.

Compound	$\lambda_{\max}$ (nm)	$\bar{\epsilon}$ (mM ⁻¹ cm ⁻¹)
Urd	262	10.1
dThd	267	9.73
NBMPR	290	25.1
NBTGR	312	16.1
Dilazep	266	18.42
Tunicamycin	259	9.4

iii. Fisher's and RPMI Transport Media (FTM, RTM)

FTM and RTM consisted of Fisher's medium and RPMI 1640 medium, respectively, buffered with 20 mM HEPES (in place of NaHCO₃) to pH 7.4. These transport medium did not contain horse or fetal bovine serum.

iv. Salts Transport Medium

These media, based on Dulbecco's salts medium (Dulbecco and Vogt, 1954), had an osmolality of 300 ± 15 mOsm (Wescor Vapor Pressure Osmometer, Model 5500) and were adjusted to pH 7.4. The sodium-free medium was prepared by the substitution of NaCl and Na₂HPO₄ with equimolar amounts of N-methyl-D-glucamine chloride (NMG-Cl) and K₂HPO₄, respectively. NMG⁺ has been used in cell transport medium as a substitute for Na⁺ (Dagnino, 1988).

Sodium-Containing Medium

1.0 mM	CaCl ₂
2.6 mM	KCl
1.4 mM	KH ₂ PO ₄
0.5 mM	MgCl ₂
5.0 mM	glucose
138.0 mM	NaCl
8.0 mM	Na ₂ HPO ₄

Sodium-Free Medium

1.0 mM	CaCl ₂
2.6 mM	KCl
1.4 mM	KH ₂ PO ₄
0.5 mM	MgCl ₂
5.0 mM	glucose
138.0 mM	NMG-Cl
8.0 mM	K ₂ HPO ₄

b) Measurement of Zero-Trans² Nucleoside Influx

Cells were harvested by centrifugation (150 x g, 5 min) and resuspended in a transport medium at 1×10^7 - 2×10^7 cells/ml. Suspensions of cells were utilized within 30 minutes of harvesting from growth medium. The composition of the cell suspension medium differed depending upon the cell line used and the type of assay. Influx assays were conducted at 37°C. Where necessary, cell density was determined by counting cells from replicate 100 μ l aliquots using a Coulter Counter. Portions (100 μ l) of transport medium containing ³H-nucleoside were layered over 200 μ l portions of transport oil in 1.5 ml microcentrifuge tubes. The tubes were placed in the rotor of a microcentrifuge (Eppendorf Models 5412 or 5414) and influx intervals were initiated by addition of 100 μ l portions of cell suspension. Influx intervals were stopped by centrifugal pelleting (9000 x g, ≥ 10 sec) of cells under the oil layer, hence this assay was termed the "oil-stop" influx assay. By adding portions of cell suspensions successively to microcentrifuge tubes placed in a single rotor, it was possible to conduct a maximum of 6 uptake assays simultaneously. Metronome signals were used to time the addition of cells

² The rate of transport of substrate (³H-nucleoside) from one face of the membrane (*cis* face) to the other (*trans* face) as measured when the concentration of substrate at the *trans* face is zero (Stein, 1986).

to each of these six tubes at intervals separated by as little as 1 sec. Measurement of influx at each interval was conducted in triplicate. Aqueous layers in the assay tubes were removed and the tube walls were washed twice with water. The oil layers were then removed and the cell pellets were dissolved in 250 μ l of 5% Triton X-100 by vortexing. The tubes were placed in scintillation vials, 5 ml xylene-detergent scintillant (Pande, 1976) was added and radioactive content was measured.

A variation of the oil-stop assay, termed the "inhibitor-stop" assay, employed an additional step wherein influx intervals were stopped by the addition of 200 μ l ice-cold transport medium containing 200 μ M dilazep dihydrochloride to the cell/ ^{3} H-nucleoside mixture, followed by immediate centrifugation. Zero-time influx was determined by rapid successive additions of ice-cold dilazep solution and cells, followed by centrifugation.

Total cell pellet and extracellular pellet volumes were determined, respectively, by substituting [^{3} H]H₂O and [^{14} C]sucrose (or [^{14} C]polyethylene glycol) for ^{3} H-nucleoside in the influx assay. Intracellular pellet volume (the difference between the total and extracellular volumes) normally constituted 80-90% of total pellet volume.

c) Inhibition of Nucleoside Influx

The effects of NT inhibitors on nucleoside influx were assessed using a procedure that minimized depletion of free inhibitor. Cells were incubated at low densities (10^5 - 10^6 cells/ml, 15-20 min) in transport medium that contained graded concentrations of inhibitor. Cells were collected by centrifugation (150 x g, 5 min) and resuspended at high densities (10^5 cells/ml) in fresh transport medium containing an identical concentration of inhibitor used in the initial incubation. Influx assays were then conducted as described above using ^{3} H-nucleoside solutions that contained inhibitor at the same concentrations as were present in the cell suspensions.

d) Calculation of Initial Rates and Kinetic Parameters

Zero-trans influx, which represents the rate of transfer of nucleoside molecules from the *cis* to the *trans* face of the plasma membrane, were calculated from (i) linear time courses of uptake by linear regression using the least squares method, and (ii) non-linear time courses by second-order polynomial regression using the least squares method (Hewlett-Packard HP-41C calculator with Stat Pac). For a single NT process, *zero-trans* fluxes reach a limiting maximum velocity as concentration increases. The relationship between influx (V) and permeant concentration (S) can be described by $V = (S \cdot V_{\max}) / (K_m + S)$ (Equation 1), wherein $V_{\max}$ and K_m represent, respectively, the maximal limiting influx and permeant concentration at the *cis* face that produces the half maximal influx (Stein, 1986). Equation 1 is mathematically identical to the Michaelis-Menten equation and kinetic parameters of influx can be estimated from linear transformations of the data according to the method of Rosenthal (1967). In cases of simultaneous expression of two kinetically distinct processes, Equation 1 can be modified to $V_t = V_1 + V_2 = [(S \cdot V_{\max 1}) / (K_{m1} + S)] + [(S \cdot V_{\max 2}) / (K_{m2} + S)]$ (Equation 2), wherein V_t is total flux, V_1 and V_2 are fluxes for process 1 and 2, $V_{\max 1}$ and $V_{\max 2}$ are maximal fluxes for processes 1 and 2, and K_{m1} and K_{m2} are the permeant concentrations at the *cis* face at half $V_{\max 1}$ and $V_{\max 2}$, respectively. Rosenthal transformation of such data, graphically represented as V_t/S versus V_t , results in a non-linear plot from which kinetic parameters can be derived by direct graphical analysis or regression analysis via computer-assisted curve fitting.

Analysis of Metabolites Arising From Salvage of Exogenous Uridine

Microcentrifuge tubes were layered, from bottom to top, with 100 μ l of 4% (v/v) perchloric acid (PCA), 200 μ l transport oil, and 100 μ l of D-[^{3} H]uridine in transport medium. Portions of cells in transport medium (100 μ l, 2×10^7 cells/ml) were added and after a 5 or 10 sec incubation at 37°C, ice-cold transport medium containing 200 μ M dilazep (200 μ l) was added to inhibit uptake of exogenous uridine. Zero-time samples were obtained by simultaneous addition of cells and the ice-cold dilazep solution. The cells were immediately pelleted by centrifugation (9000 x g, 10 sec), the aqueous and oil layers were removed by aspiration, and the cells were resuspended in the 4% PCA and incubated on ice for 20 min. Extracts containing cellular nucleobases, nucleosides, and nucleotides were prepared from the PCA fraction at room temperature by the method of Khym (1975). The PCA fractions were neutralized with an equal volume of 0.5 M Alamine 336 in Freon-TF by gently mixing for 4 min, and the upper aqueous phase was recovered after centrifugation (9000 x g, 4 min). Equal volumes of aqueous phase were immediately loaded onto cellulose sheets and subjected to ascending thin layer chromatography (TLC) using a solvent system of isopropanol/saturated ammonium sulfate/H₂O (2:79:19 v/v). This solvent system effectively resolved uracil (R_f = 0.52), uridine (R_f = 0.62), and uridine 5'-phosphates (UMP, UDP, UTP; R_f = 0.68 to 0.82). The TLC sheets were cut into 1 cm wide lanes, dissolved in 5 ml xylene-detergent scintillant (Pande, 1976), and subjected to liquid scintillation counting. Radioactivity migrating as uracil, uridine, or uridine 5'-phosphorylated metabolites was calculated as a percentage of the total radioactivity loaded.

Equilibrium Binding of [^{3} H]NBMPR to Intact Cells

Binding of NBMPR to cultured cells was determined using a modification of an established method (Cass *et al.*, 1981). Cells were harvested by centrifugation (150 x g, 5 min) and washed once and resuspended in transport medium at 2×10^6 cells/ml. Portions (500 μ l) of cell suspension were added to 500 μ l of transport medium containing graded concentrations of [^{3} H]NBMPR alone or with 20 μ M nitrobenzylthioguanosine (NBTGR) that had been layered over 250 μ l of transport oil in microcentrifuge tubes. The mixtures were incubated 20-30 min at either RT or 37°C. Cells were centrifugally pelleted and samples were processed thereafter as described for influx assays. The concentration of free NBMPR was estimated from the radioactive content of the aqueous medium after centrifugation and the quantity of total and non-specifically bound NBMPR was determined from the radioactive content of cell pellets incubated in the absence and presence of NBTGR, respectively. Site-specific binding of NBMPR was calculated as the difference between total and non-specific binding. The site-specific binding data were subjected to Rosenthal transformation (Rosenthal, 1967) and values for the maximal number of binding sites (B_{max}) and the dissociation constant (K_d) were calculated by linear regression analysis.

Isolation of Crude Membrane Fractions

Cells were collected by centrifugation (150 x g, 10 min), washed twice in phosphate-buffered saline (PBS; 137 mM NaCl, 5.36 mM KCl, 1.1 mM KH₂PO₄, 1.1 mM Na₂HPO₄, pH 7.2) and resuspended in lysis buffer (150 mM NaCl, 20 mM HEPES, 2 mM MgCl₂, 1 mM EDTA, pH 7.1) at 2×10^6 to 3×10^6 cells/ml (Ross *et al.*, 1977). These and all subsequent steps were carried out at 4°C. The cells were disrupted by sonication (3 x 10 sec pulses) with a Braunsonic 1510 sonicator and the cell lysate was centrifuged (1500 x g, 5 min). The resulting supernatant was further centrifuged (30000 x g, 30 min). Pellets containing crude membranes were used immediately (as described below) or resuspended in 20 mM Tris-HCl (pH 7.2) and stored under liquid nitrogen.

Enrichment for Plasma Membranes

Fractions enriched for plasma membranes were prepared from crude membranes using a procedure similar to that described by Belsham *et al.* (1980). All procedures were carried out at 4°C. The crude membranes were suspended in Tris-sucrose buffer (0.5 M sucrose, 4 mM EGTA, 20 mM Tris-HCl pH 7.4) and subjected to Dounce homogenization (30 strokes). The homogenate was layered onto Percoll (17.5%, v/v) in Tris-sucrose buffer and centrifuged (20000 x g, 25 min). These conditions produced Percoll-generated density gradients of between 1.035-1.070 g/ml (Boleti, 1991). The plasma membrane fraction, isolated as a thin layer at the top of the gradient, was collected by centrifugation (100000 x g, 10 min) and washed with PBS to remove the Percoll. Enriched plasma membrane fractions were suspended in 20 mM Tris-HCl (pH 7.2) and stored under liquid nitrogen.

Photoaffinity Labeling of Membrane Proteins With [³H]NBMPR

Plasma membrane fractions were quantitated by the bicinchoninic acid (BCA) protein assay (Smith *et al.*, 1985) and photoaffinity labeled under equilibrium binding conditions with [³H]NBMPR using an established method (Gati *et al.*, 1986) with modifications. Plasma membrane fractions (250 µg protein), prepared as described above, were suspended at 4°C in photolabeling buffer (10 mM dithiothreitol, 150 mM NaCl, 20 mM HEPES, 2 mM MgCl₂, 1 mM EDTA, pH 7.1) that contained 100 nM [³H]NBMPR alone or with 10 µM NBTGR. The mixtures were incubated at RT for 30 min in Beckmann 5 x 20-mm centrifuge tubes and then chilled to 4°C and placed on ice at a distance of 4 cm from the cooling sleeve of a Hanovia 450-W mercury arc lamp. The membrane mixture was irradiated for 60 sec, diluted 5-fold with photolabeling buffer that contained 20 µM NBTGR, and incubated 15 min at RT. Membranes were recovered by centrifugation (40000 x g, 30 min, 4°C) and were used immediately or were suspended in 20 mM Tris HCl (pH 7.2) and stored at -70°C.

Deglycosylation of [³H]NBMPR-Photolabeled Membrane Proteins

a) Enzymatic Cleavage of N-linked Oligosaccharides

Two alternate enzymatic procedures were used, according to manufacturers' instructions, to deglycosylate membrane proteins. Photolabeled membranes (200-300 µg protein) were solubilized in buffer (100 mM NaH₂PO₄, 50 mM EDTA, 0.5% Nonidet P-40 (v/v), 0.1% SDS (w/v), 1.0% 2-mercaptoethanol (v/v), pH 6.1) and incubated (17 hr, 37°C) with endoglycosidase F at a concentration of 6 U/mg protein. Alternatively, the membranes (200-300 µg protein) were solubilized in buffer (120 mM NaH₂PO₄, 10 mM 1,10-phenanthroline hydrate, 2.0% Nonidet P-40 (v/v), 0.15% SDS (w/v), 0.5% 2-mercaptoethanol (v/v), pH 8.6) and incubated (17 hr, 37°C) with N-glycanase at a concentration of 6 U/mg protein.

b) Enzymatic Cleavage of O-Linked Oligosaccharides

Membrane proteins were subjected to enzymatic digestion according to the manufacturers' instructions. Photolabeled membranes (200-300 µg protein) were suspended in 100 mM CH₃COONa/19 mM KH₂PO₄ (pH 6.5) and sequentially incubated with 0.5 U neuraminidase (18 hr, 22°C), and then with O-glycanase for 24 hr at 22°C.

c) Chemical Cleavage of N- and O-Linked Oligosaccharides

Chemical hydrolysis of Ser/Thr-linked and Asn-linked carbohydrate moieties was performed using trifluoromethanesulfonic acid (TFMS) as previously described (Kaylan and Bahl, 1983). Photolabeled membranes (0.5 mg protein) were placed in a glass vial (with Teflon-lined screw cap), frozen using dry ice/acetone, and lyophilized. The pellet was resuspended in 0.5 ml of 1:2 anisole/TFMS (v/v) and the solution was purged with

N₂ for 1 min. The vial was sealed and incubated for 2.5 hr at 4°C. The hydrolysis reaction was terminated by neutralizing the mixture with 9 ml of 1:1 pyridine/H₂O (v/v) that had been cooled in a dry ice/acetone bath. The solution was then dialyzed extensively against dH₂O at 4°C and membranes were collected by centrifugation (35000 x g, 35 min, 4°C).

Gel Electrophoresis of [³H]NBMPR-Photolabeled Membrane Proteins

All SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analyses used in this study employed the method described by Laemmli (1970). Samples were solubilized in an equal volume of protein sample loading buffer (4% SDS (w/v), 20% glycerol (v/v), 2% 2-mercaptoethanol (v/v), 0.01% bromophenol blue (w/v), 20 mM Tris-HCl pH 6.8). The samples were electrophoretically separated on slab gels (BioRad Protean II or MiniProtein II apparatus) using a 4% acrylamide stacking gel and separating gel of 12% acrylamide concentration. Gels were stained with Coomassie stain (0.05% Coomassie blue (w/v), 25% isopropanol (v/v), 10% acetic acid (v/v)) and destained (5% methanol/10% acetic acid) to visualize protein. In the case of [³H]NBMPR-photolabeled samples, the lanes to which ³H-labeled samples had been added were cut into 2 mm slices (Hoeffer Gel Slicer) and individual slices were placed in scintillation vials. Gel slices were solubilized in 2 ml of 3% Protosol in Econofluor and subjected to liquid scintillation counting. The relative mobility of [³H]NBMPR-labeled protein was ascertained by comparison to protein molecular weight standards (Sigma Dalton Mark VII-L: bovine albumin, 66000; egg albumin, 45000; rabbit glyceraldehyde-3-phosphate dehydrogenase, 36000; bovine carbonic anhydrase, 29000; bovine trypsinogen, 24000; soybean trypsin inhibitor, 20100; bovine α -lactalbumin, 14200; Pharmacia LMW Standards: rabbit phosphorylase b, 94000; rabbit albumin, 67000; egg ovalbumin, 43000; bovine carbonic anhydrase, 30000; soybean trypsin inhibitor, 20100; bovine α -lactalbumin, 14400) that were run in adjacent lanes.

Measurement of Plasma Membrane N-Linked Glycoprotein Turnover

Pulse-chase experiments to measure the general turnover of N-linked glycoproteins employed cells that had been metabolically labeled with D-[³H]mannose for 45 hr. These cells were collected by centrifugation (150 x g, 10 min), washed twice with PBS, resuspended in [³H]mannose-free growth media, and allowed to proliferate in the presence or absence of 2 μ M tunicamycin. Aliquots of cells were harvested at intervals thereafter and plasma membrane-enriched fractions were prepared for analysis by SDS-polyacrylamide electrophoresis. Lanes containing ³H-labeled samples were cut into 2-mm slices and radioactivity associated with each slice was measured as described above.

RESULTS

Selection of Conditions For Exposure of L1210/C2 Cells to Tunicamycin

Since tunicamycin had previously been shown to be growth inhibitory to L1210 cells (Morin *et al.*, 1983), experiments were undertaken to identify exposure conditions that inhibited synthesis of N-linked oligosaccharides without major toxicity to cells. The measurement of cell population increase over time in the presence of varying concentrations of a drug has been shown to reflect the relative toxicity of that drug (Cass *et al.*, 1979). The growth-inhibitory effects of increasing concentrations of tunicamycin on L1210/C2 cells are shown in Fig. II 4. The concentrations of tunicamycin that

inhibited proliferative rates by 50% (IC₅₀) after 48 and 72 hr exposures were 1.3 and 1.6 μ M, respectively. Minimal toxicity, indicated by only minor antiproliferative effects, was observed during the first 24 hr of exposure at concentrations of tunicamycin up to 3 μ M.

The incorporation of [³H]thymidine, [³H]leucine, or [³H]mannose into acid-insoluble cellular extracts of L1210/C2 cells grown in increasing concentrations of tunicamycin was measured to assess the effects of a 24 hr exposure to tunicamycin on the overall synthesis of nucleic acids, protein, and N-linked oligosaccharides, respectively. Fig. II 5 shows that exposure of L1210/C2 cells to 2 μ M tunicamycin reduced the incorporation of [³H]mannose and [³H]leucine by 60-70% and 15-25%, respectively, and increased [³H]thymidine incorporation by 15-30%. The stimulation of [³H]thymidine incorporation by tunicamycin has been previously reported (Morin *et. al.*, 1983), however the mechanism responsible is unknown. Concentrations of tunicamycin less than 2 μ M inhibited the incorporation of [³H]mannose only slightly, whereas concentrations higher than 2 μ M led to decreased incorporation of [³H]leucine. Therefore, a standard condition of exposure to 2 μ M tunicamycin over a 24 hr period was selected as one that reduced synthesis of N-linked oligosaccharide without also having major effects on protein or DNA synthesis. Growth in this exposure condition, as compared to growth in the absence of tunicamycin, was inhibited, as reflected by an increase of the population doubling time from 14 ± 1 to 23 ± 3 hr. It was also noted that cells became increasingly fragile after exposure to 2 μ M tunicamycin for longer than 24 hr.

The tunicamycin-related inhibition of mannose incorporation into total cellular protein shown in Fig. II 5 may not be representative of effects on plasma membrane glycoproteins since different cellular compartments contain proteins that exhibit varying periods of residency and rates of degradation. The effects of tunicamycin exposure (2 μ M, 24 hr) on the incorporation of [³H]mannose into newly synthesized plasma membrane glycoproteins are illustrated in Fig. II 6. Plasma membrane-enriched fractions prepared from L1210/C2 cells grown with [³H]mannose in the presence or absence of tunicamycin were subjected to SDS-polyacrylamide electrophoresis and radioactivity associated with gel slices from each lane of sample was measured. Cells grown in the absence of tunicamycin exhibited substantial incorporation of [³H]mannose into glycoproteins, especially those larger than 30 kDa. Exposure to tunicamycin produced near complete inhibition of this incorporation into all plasma membrane glycoproteins, suggesting that the addition of N-linked oligosaccharide to plasma membrane proteins had been effectively blocked by the exposure conditions.

A pulse-chase experiment was performed to examine the effects of tunicamycin exposure on the turnover of pre-existing plasma membrane glycoproteins. Plasma membrane glycoproteins were [³H]mannose-labeled by growth of L1210/C2 cells with [³H]mannose for 3 generations (45 hr), after which the cells were cultured without mannose in the presence or absence of 2 μ M tunicamycin. The radioactivity associated with plasma membrane-enriched fractions prepared from cells after 0, 12, and 24 hr of growth in the absence or presence of tunicamycin is shown in Fig. II 7. Although the radioactive content was slightly higher in tunicamycin-treated cells after 24 hr of exposure, the half-life of [³H]mannose-labeled plasma membrane glycoproteins was approximately equal (19-21 hr) in cells grown either in the presence or absence of tunicamycin. It appears that tunicamycin exposure did not appreciably alter the general turnover of N-linked glycoproteins that reside in the plasma membrane.

Collectively, the results shown in Figs. II 4-7 indicate that a 24 hr exposure of actively proliferating L1210/C2 cells to 2 μ M tunicamycin provided almost complete inhibition of N-linked glycosylation of newly synthesized plasma membrane proteins with minimal disruption of cell proliferation and general protein synthesis/degradation.

Use of a Inhibitor-Stop Method to Measure Initial Rates of Transport

Nucleoside influx studies were conducted at 37°C with L1210/C2 cells to determine kinetic parameters of nucleoside transport processes under normal physiological conditions. Uridine was employed as a nucleoside permeant since it had been demonstrated to be a permeant for the *es* and *ei* NT processes present in L1210/C2 cells (Belt, 1983). Preliminary results of studies carried out at 37°C using the oil-stop method revealed non-linear time courses that evidently reached non-linearity during the period of centrifugation required to remove cells from the ^3H -nucleoside solution. In an earlier study (Harley *et al.*, 1982), it was shown that influx assays terminated by this method require approximately 2 sec to remove cells from the ^3H -nucleoside solution. Under these circumstances, the degree of nucleoside association with cells at time zero cannot be directly measured. Since precise measurements of initial rates of transport require time courses that exhibit linearity and/or a true measurement of influx at time zero, inhibitors of NT processes, such as NBMPR and dilazep, have been used to "instantaneously" terminate nucleoside influx (Harley *et al.*, 1982; Paterson *et al.*, 1984).

Dilazep was employed as the inhibitor-stop agent in this study because of its high solubility in aqueous solution and capacity for inhibition of equilibrative NT processes (Paterson *et al.*, 1984; Plagemann and Kraupp, 1986). Fig. II 8 shows the inhibition of uridine influx after addition of either 4° or 37°C transport medium containing 200 μM dilazep dihydrochloride to cell/ ^3H -nucleoside mixtures. The assay mixtures were allowed to incubate in the microfuge for periods of time prior to centrifugation to assess the effectiveness of the inhibitor-stop procedure. For comparison, a time course of influx generated using the oil-stop method is also presented. Influx in the presence of both low (11 μM) and high (382 μM) concentrations of exogenous uridine (as well as efflux of internalized uridine) was completely inhibited for a period of approximately 4 sec following the addition of 4°C dilazep solution. Influx was observed after this 4 sec period, presumably due to the warming of the cell/ ^3H -nucleoside/dilazep mixture. This assumption is supported by the observation that although addition of 37°C dilazep solution initially inhibited uridine flux, influx was re-initiated immediately following its addition. The effectiveness of the inhibitor-stop procedure was therefore dependent upon the combination of decreased temperature of the assay mixture upon addition of ice-cold solution and the transport-inhibitory action of dilazep.

Metabolism of Internalized Uridine During Influx Assays

The metabolism and subsequent efflux of intracellularized permeant during the measurement of zero-*trans* influx of the permeant can give rise to misleading time courses of influx. Intracellular uridine can be rapidly converted to either phosphorylated intermediates (UMP, UDP, UTP) or uracil in L1210 cells (Roobol *et al.*, 1984) and other cultured cells (Wohlheuter and Plagemann, 1980). Flux of phosphorylated uridine intermediates across the plasma membrane has not been documented, presumably due to the absence of nucleotide-selective transport process and low rates of passive diffusion of charged molecules. Uracil-selective transport processes are present in the plasma membranes of many cultured mammalian cells (Marz *et al.*, 1979) and may mediate efflux in the presence of high intracellular concentrations of uracil. Conversion of intracellular uridine into uracil in L1210/C2 cells may occur rapidly at 37°C, possibly leading to efflux of uracil and subsequent invalidation of zero-*trans* influx measurements.

The intracellular metabolism of [^3H]uridine by L1210/C2 cells was examined over a 0-10 sec period during zero-*trans* influx assays and the results of two such experiments conducted with either low (8 μM) or high (378 μM) concentrations of [^3H]uridine are shown in Fig. II 9. A normal influx assay using the inhibitor-stop method was employed, except that cells were spun into 4% PCA that had been layered under the

transport oil. The PCA-soluble fractions were analyzed for [^3H]uracil, [^3H]uridine, and phosphorylated [^3H]uridine intermediates (UMP, UDP, UTP) by TLC. In the experiment with the low concentration of uridine, production of uracil from intracellular uridine during influx assays constituted less than 3% of ^3H -label present and the majority of internalized uridine ($\approx 90\%$) had been converted to phosphorylated intermediates after 10 sec. In the experiment with the high concentration of uridine, uracil accounted for 5, 11, and 15% of total intracellular ^3H -label after 0, 5, and 10 sec, respectively. While a small quantity of uridine was evidently intracellular at the time zero assay points, the true extent of uridine metabolism was probably over-estimated since metabolism continued during centrifugation of cells through the oil layer. It was evident that uridine metabolism occurred over longer periods of time, therefore this study used zero-*trans* influx assays with an inhibitor-stop over a maximum period of 0-6 sec.

Exposure to Tunicamycin Alters the Functional Expression of Nucleoside Transport Processes in L1210/C2 Cells

The role of N-linked oligosaccharide in the function of NT processes was addressed by examining the effects of tunicamycin exposure on the kinetics of uridine transport in L1210/C2 cells. The kinetic parameters of a transport process can be ascertained through the measurement of transport rates as a function of permeant concentrations. Accordingly, the concentration-dependence of zero-*trans* influx of [^3H]uridine was measured in L1210/C2 cells that had been grown in the presence or absence of 2 μM tunicamycin for a 24 hr period. Representative time courses generated using L1210/C2 cells grown in the absence or presence of tunicamycin are shown in Fig. II 10 and Fig. II 11, respectively.

The rates for zero-*trans* influx of uridine were calculated from time courses (see Materials and Methods) generated using untreated and tunicamycin-treated cells and were plotted as a function of uridine concentration. Results from such experiments, an example of which is illustrated in Fig. II 12, revealed that the velocity of influx in untreated and treated cells was nearly identical at uridine concentrations less than 50 μM . However, treated cells exhibited a 2-3 fold increase in velocities at higher concentrations. It is interesting to note that untreated cells exhibited a linear relationship between transport velocity and uridine concentration, whereas tunicamycin-treated cells exhibited an apparent saturation of transport at high uridine concentrations.

Tunicamycin is a nucleoside analog and may have interfered with uridine fluxes in tunicamycin-treated cells since residual tunicamycin ($< 0.1 \mu\text{M}$) may have been present during transport assays. However, the initial rates of zero-*trans* influx of 1.3 μM uridine measured in the absence or presence of 4 μM tunicamycin were 0.134 ± 0.005 and 0.141 ± 0.016 pmol/ μl cell $\text{H}_2\text{O}\cdot\text{sec}$, respectively, indicating that tunicamycin itself was not contributing towards the altered transport behavior. It was also possible that tunicamycin treatment induced a greater degree of passive diffusion. The cell pellet volume that was accessible to [^{14}C]sucrose (extracellular volume) would have increased if the tunicamycin-treated cells had become leaky. Values (mean $\pm$ SD for 8 separate experiments) for the extracellular volume in untreated and tunicamycin-treated cells were $14.3 \pm 2.0\%$ and $12.2 \pm 1.6\%$, respectively. Thus, no differences in general permeability between treated and untreated cells were observed.

The results shown in Fig. II 12 were subjected to Rosenthal transformation (Rosenthal, 1967) for determination of the kinetic parameters (K_m , V_{max}) of zero-*trans* influx. Graphical representation of this transformation shown in Fig. II 13 revealed the apparent presence of both high- and low-affinity transport activities in untreated and

tunicamycin-treated L1210/C2 cells³. The kinetic parameters of the high- and low-affinity activities from three experiments were graphically derived, albeit with an overestimation of true values, as described by Bylund (1980) and are presented in Table II 1. The estimated K_m and V_{max} values (mean $\pm$ SD for three separate experiments) of the high-affinity transport activity in untreated and tunicamycin-treated cells were 41 ± 10 and 46 ± 21 μ M, and 8 ± 3 and 11 ± 4 pmol/ μ l cell H₂O·sec, respectively. The estimated K_m and V_{max} values of the low-affinity transport activity in tunicamycin-treated cells were 366 ± 26 μ M and 191 ± 34 pmol/ μ l cell H₂O·sec, respectively. Kinetic parameters of low-affinity transport in untreated cells could not be graphically estimated due to the apparent non-saturable nature of the transport process(es) responsible, at least as determined under the experimental conditions employed in this study. Thus, although tunicamycin treatment had little or no effect on the kinetic behavior of high-affinity transport activity, it induced decreases in the K_m and V_{max} values of low-affinity transport. Transport processes responsible for low-affinity transport activity must therefore be sensitive to the action of tunicamycin.

The Sensitivity of Nucleoside Transport to Inhibition by NBMPR is Increased in Tunicamycin-Exposed L1210/C2 Cells Without an Increase in the Number of NBMPR-Binding Sites

The effects of NBMPR on uridine transport in untreated and tunicamycin-treated cells was assessed to determine whether exposure to tunicamycin altered the relative proportions of NBMPR-sensitive and -insensitive transport activity. Results shown in Fig. II 14 reveal that the majority of *zero-trans* influx of uridine in both treated and untreated cells was sensitive to inhibition by NBMPR, as measured at both high and low concentrations of uridine. The IC₅₀ value of NBMPR-sensitive transport in L1210/C2 cells grown in the absence or presence of 2 μ M tunicamycin for 24 hr was similar (0.8-1.5 nM). However, transport of uridine in tunicamycin-treated cells appeared to be somewhat more sensitive to inhibition by the higher concentrations (>10 nM) of NBMPR than in untreated cells at both the low (1 μ M) and high (300 μ M) concentrations of uridine. These results suggested that tunicamycin-treated cells either expressed a greater number of NBMPR-sensitive transporters or the contribution of NBMPR-insensitive transport processes towards overall transport was significantly decreased.

The presence of high-affinity NBMPR-binding sites has been correlated with the presence of NBMPR-sensitive transport processes in cultured cells (Cass *et al.*, 1974; Cass *et al.*, 1981). NBMPR-binding sites were quantitated in cells grown in the absence or presence of tunicamycin (2 μ M, 24 hr) to ascertain whether the number of NBMPR-sensitive transporters increased upon exposure to tunicamycin. The saturable nature of NBMPR binding to L1210/C2 cells is illustrated in Fig. II 15. Specific binding of NBMPR to transport-inhibitory sites, which was determined by the capacity of the structural analog NBTGR to block binding of NBMPR, was also saturable. The amounts of specifically and non-specifically bound NBMPR were identical in untreated and tunicamycin-treated cells. Binding constants for specific NBMPR binding in treated and untreated cells, as measured under several conditions, are shown in Table II 2. Treated and untreated L1210/C2 cells expressed approximately 2.2×10^5 NBMPR-binding sites/cell at 37°C, with K_d values of approximately 0.25 nM. These K_d values were similar to the IC₅₀ values (0.8-1.5 nM) for NBMPR inhibition of uridine transport,

³ The determination of K_m and V_{max} values for two kinetically distinct transport processes is possible, although, during the course of this study the existence of a third uridine-selective (*cif*) nucleoside transport process was demonstrated in L1210/C2 cells (Dagnino, 1988; Dagnino and Paterson, 1990). Since the three nucleoside transport processes of L1210/C2 cells were functionally expressed as only two kinetically distinguishable activities, exact kinetic parameters for each transport process could not be assigned.

consistent with the conclusion that NBMPR binding to its high-affinity sites was responsible for inhibition of transport by NBMPR.

Binding studies were also conducted at RT (20°C) with untreated and tunicamycin-treated cells. The K_d values (0.13 nM) obtained at RT were 50% less in both untreated and treated cells than those observed at 37°C. A temperature-dependent shift in K_d values has also been observed in K562 cells and HeLa cells (Boleti, 1991; Lauzon and Paterson, 1977).

The NBMPR binding studies revealed that the apparent increase in NBMPR-sensitive transport activity observed in tunicamycin-treated cells was not a result of increased numbers of NBMPR-sensitive transporters. An alternate explanation for the overall increase in the proportion of NBMPR-sensitive transport activity may be a decrease in the proportion of NBMPR-insensitive transport activity, an activity that is mediated by the *ei* and *cif* NT processes present in L1210/C2 cells.

Exposure to Tunicamycin Confers Increased Functional Expression of the *es* NT Process and Decreased Functional Expression of the Na^+ -Dependent (*cif*) NT Process in L1210/C2 Cells

The relative contributions of the NBMPR-sensitive, NBMPR-insensitive, and Na^+ -dependent transport processes towards the total transport exhibited by L1210/C2 cells were examined by conducting zero-trans influx assays in (i) the presence of Na^+ and absence of 10 μM NBMPR (total transport activity), (ii) the presence of both Na^+ and 10 μM NBMPR (NBMPR-insensitive transport activity), and (iii) the absence of Na^+ and presence of 10 μM NBMPR (Na^+ -independent, NBMPR-insensitive transport activity). Time courses for the influx of 10 μM [^3H]uridine determined under these conditions, for cells grown in absence or presence of 2 μM tunicamycin for 24 hr, are illustrated in Fig. II 16. These transport assays employed 10 μM [^3H]uridine, a concentration observed to be below the apparent K_m values of listed in Table II 1, in order that transport processes not be operating at rate-limiting capacity. In addition, little difference was noted in transport rates between tunicamycin-treated and untreated cells at this concentration (Fig. II 12).

Initial rates of uridine transport derived from the time courses of Fig. II 16 and the contribution of each component to total transport are listed in Table II 3. Tunicamycin-treated and untreated cells exhibited initial rates of total uridine transport, representing all transport processes present, that differed only by 5% (2.10 versus 2.32 pmol/ μl cell $\text{H}_2\text{O}\cdot\text{sec}$, respectively). Similarly, the rates attributed to the NBMPR-insensitive, Na^+ -independent (*ei*) process differed by less than 5% between treated and untreated cells (0.21 versus 0.22 pmol/ μl cell $\text{H}_2\text{O}\cdot\text{sec}$, respectively). However, the component mediated by the Na^+ -dependent, NBMPR-insensitive (*cif*) transport process was drastically reduced in tunicamycin-treated (0.27 pmol/ μl cell $\text{H}_2\text{O}\cdot\text{sec}$) compared to untreated cells (0.82 pmol/ μl cell $\text{H}_2\text{O}\cdot\text{sec}$), suggesting that the tunicamycin exposure selectively decreased the functional expression of the *cif* NT process in L1210/C2 cells. Interestingly, the contribution of the NBMPR-sensitive (*es*) transport process towards total uridine influx was increased by about the same amount that Na^+ -dependent transport was decreased.

Increased *es* NT activity was not accompanied by increased numbers of NBMPR-binding sites (Table II 2), suggesting that the *es* NT process had been somehow "activated". The mechanism by which the tunicamycin-treated cells differentially altered the activities of these two NT processes could not be explained by the results of these transport studies.

The *es* NT Protein of L1210/C2 Cells Contains N-Linked Oligosaccharides

Site-specific photolabeling with [^3H]NBMPR has been previously used to identify membrane proteins associated with equilibrative NT in erythrocytes and cultured mammalian cells (Young *et al.*, 1983; Young *et al.*, 1984). In the experiments of Fig. II 17, NBMPR-binding proteins were identified in the plasma membranes of L1210/C2 cells by UV-photoaffinity labeling with [^3H]NBMPR. Labeled proteins (Fig. II 17, top panel) migrated as a single broad peak of 48-55 kDa and photolabeling was abolished in the presence of excess NBTGR, a structurally related inhibitor of NT. The results of photolabeling of plasma membranes prepared from L1210/C2 cells grown in the presence of 2 μM tunicamycin for 24 hr are shown in Fig. II 17 (bottom panel). Inhibition of N-linked glycosylation by tunicamycin induced the formation of a second photolabeled peak of 42-48 kDa, suggesting that deglycosylated *es* NT protein constituted a small, yet significant, proportion of total *es* NT protein present after tunicamycin exposure.

The effects of a longer tunicamycin exposure were examined to determine if the plasma membrane *es* NT protein could be completely converted to the lower molecular weight deglycosylated form. L1210/C2 cells were grown over a 72 hr period in the presence of 0.8 μM tunicamycin, a concentration that exerted minimal antiproliferative effects over a longer period of time (see Fig. II 4). [^3H]NBMPR photolabeling of plasma membranes prepared after 36, 50, and 72 hr exposures to tunicamycin revealed the concurrent appearance of the 42-47 kDa ^3H -labeled peak and disappearance of the 48-55 kDa peak (Fig. II 18). The complete disappearance of the 48-55 kDa peak did not result in the formation of a proportionally similar 42-47 kDa labeled peak after the 72 hr exposure, suggesting that some photolabeling sites were not accessible after prolonged exposure to tunicamycin.

To assess whether deglycosylation induced by prolonged exposure to tunicamycin altered the quantity of *es* NT proteins in L1210/C2 cells, the number of high-affinity NBMPR binding sites was measured in cells grown in the presence or absence of 0.8 μM tunicamycin for 50 and 72 hr. Values presented in Table II 4 show that after a 72 exposure, tunicamycin-treated and untreated cells expressed 0.97×10^5 sites/cell and 0.90×10^5 sites/cell, respectively. NBMPR is a lipophilic molecule and can readily permeate into cells and bind to intracellular high-affinity binding sites, in addition to sites present in the plasma membrane. The photolabeling studies were conducted using plasma membrane preparations and reflect only NBMPR-binding sites present in plasma membrane. The number of NBMPR-binding sites was similar in both tunicamycin-treated and untreated cells, suggesting that the discrepancy between the NBMPR binding and photolabeling studies may be due to retention of NBMPR-binding (*es* NT) proteins in the intracellular compartment.

A successive decrease in numbers of NBMPR-binding sites in both treated and untreated L1210/C2 cells over prolonged culture periods was observed, as indicated in Tables II 1 and 3. Binding values declined from 2.2×10^5 sites/cell at 24 hr to 1.34×10^5 sites/cell at 50 hr and 0.97×10^5 sites/cell at 72 hr. During this period, the culture concentrations increased from approximately 3.0×10^5 to 10.0×10^5 cells/ml and cultures had approached stationary phase. This growth-dependent expression of NBMPR-binding sites has previously been observed in HeLa cells (Cass *et al.*, 1979) where it has been linked to regulated expression of *es* NT during the cell cycle.

N-Linked Oligosaccharide of the *es* NT Protein of L1210/C2 Cells is of the Hybrid and/or Multiple Antennary Complex Types

N-linked oligosaccharide chains of glycoproteins are classified into variations of 3 basic types: high-mannose type, multiantennary complex-type, and hybrid-type. The type and extent of N-linked oligosaccharide chains, and the possible presence of O-linked oligosaccharide chains, on the *es* NT protein of L1210/C2 cells was addressed by

endoglycosidase and chemical hydrolysis studies of [^3H]NBMPR photolabeled plasma membrane fractions.

O-Linked, as opposed to N-linked, glycoproteins contain oligosaccharide chains attached at serine or threonine residues via a gal- β -(1,3)galNAc core disaccharide structure (for review, see Hart, 1992). To ascertain whether the *es* NT protein contained O-linked oligosaccharide, [^3H]NBMPR photolabeled plasma membrane fractions were digested with endo- α -N-acetyl-galactosaminidase (O-glycanase), an endoglycosidase that hydrolyzes the gal- β -(1,3)galNAc core disaccharide linkage. [^3H]NBMPR photolabeled plasma membrane fractions prepared from L1210/C2 cells were incubated with neuraminidase prior to O-glycanase digestion to remove any galactosyl or N-acetylgalactosaminyl residues that may be present and that would interfere with O-glycanase hydrolysis. Shown in Fig. II 19 are the results of a typical control reaction that lacked endoglycosidase (bottom panel) and a sequential digestion of [^3H]NBMPR photolabeled plasma membrane with neuraminidase and O-glycanase (top panel). The appearance of a single ^3H -labeled peak of 48-55 kDa, identical to that observed for non-digested sample, suggests that little or no oligosaccharide is linked to the *es* NT protein via serine or threonine residues.

Endoglycosidase F preferentially cleaves only the di-N-acetylchitobiose moiety of high-mannose and biantennary complex-type oligosaccharide chains, whereas peptide-N ^{4} [N-acetyl- β -glucosaminyl]asparagine amidase (N-glycanase) cleaves the adjacent N-acetylglucosaminylasparagine linkage of all types of N-linked oligosaccharide chain (Nageswara and Bahl, 1987). N-Glycanase is, however, incapable of N-acetylglucosaminylasparagine linkage hydrolysis at either a C- or N-terminal asparagine. The bottom panel of Fig. II 20 illustrates the results of endoglycosidase F digestion of [^3H]NBMPR photolabeled plasma membrane. Little or no hydrolysis of N-linked oligosaccharide chain was suggested by the presence of the 48-55 kDa ^3H -labeled peak. The results of N-glycanase digestion shown in the top panel of Fig. II 20 revealed the presence of a smaller 41-46 kDa ^3H -labeled peak. The apparent susceptibility of the N-linked oligosaccharide chains of [^3H]NBMPR-labeled *es* NT protein to hydrolysis by N-glycanase, but not endoglycosidase F, indicated that the oligosaccharide moiety was of a tri- or tetraantennary complex type and/or hybrid type.

N-glycanase digestion (Fig. II 20) and tunicamycin treatment (Fig. II 18) revealed that glycosylation accounts for 7-9 kDa (15-16%) of the total molecular weight of the *es* NT protein in L1210/C2 cells. To confirm these observations, chemical hydrolysis of [^3H]NBMPR photolabeled plasma membrane using trifluoromethanesulfonic acid (TFMS) was performed. TFMS cleaves both N- and O-glycosidic linkages of glycoproteins (Hakimuddin and Bahl, 1987). Hydrolysis with TFMS, illustrated in Fig. II 21, resulted in the formation of smaller species of ^3H -labeled peaks of 31-46 kDa. These ^3H -labeled peaks were similar to those observed after N-glycanase digestion and tunicamycin treatment. The conditions of TFMS treatment may have also given rise to hydrolytic fragments of ^3H -labeled *es* NT protein evident as a component(s) of these species. The poor recovery of [^3H]NBMPR photolabeled protein after TFMS treatment, evident in Fig. II 21 and observed in other TFMS hydrolysis experiments, supports this assumption. The results of the chemical hydrolysis experiments confirm the extent of glycosylation present on *es* NT protein of L1210/C2 cells.

DISCUSSION

The results presented in this chapter demonstrate that N-linked glycosylation is required for the proper function and/or expression of the *cif* NT protein of L1210/C2 cells. The extent and structure of the N-linked oligosaccharide moiety associated with the *es* NT protein, as well as a possible functional role for this moiety in the transport process, has also been addressed in this study.

A functional assessment of NT processes in L1210/C2 cells was performed by measurement of uridine influx at 37°C. Accurate measurements of zero-*trans* uridine influx at 37°C were obtained using a combination of (i) a short (0-6 sec) time course, that limited any potential influence that intracellular uridine metabolism may have had upon the measurements, and (ii) a 4°C dilazep stop followed by immediate separation of cells from nucleoside-containing medium. Instantaneous inhibition of uridine fluxes by the inhibitor stop method can be explained by the sensitivity of equilibrative NT processes to inhibition by dilazep and the decreased activity of the *es* (Plagemann and Wohlheuter, 1984) and *cif* NT processes (Dagnino, 1988) observed at lowered temperatures. The 4°C dilazep stop has also been used to examine nucleoside transport at 37°C in human K562 erythroleukemia cells (Boleti, 1991).

Approximately 70-85% of total transport in L1210/C2 cells, measured at either high (300 µM) or low (1 µM) concentrations of uridine, was highly sensitive to inhibition by nanomolar concentrations of NBMPR and gave rise to IC₅₀ values of 0.8-1.0 nM. The presence of NBMPR-sensitive uridine influx was presumed to be due to occupancy of high-affinity binding sites associated with *es* NT proteins, since specifically-bound NBMPR exhibited K_d values (0.25 nM) similar to the IC₅₀ values for NBMPR inhibition of influx. The residual transport, which was insensitive to inhibition by 10 µM NBMPR, was due to the combined activities of the *ei* and *cif* NT processes.

In an earlier study conducted at RT, L1210/C2 cells were shown to express *cif* NT (Dagnino, 1988), in addition to *es* and *ei* NT processes (Belt, 1983). In the current study, the percentage of total transport of 10 µM uridine in L1210/C2 cells at 37°C mediated by *es*, *ei*, and *cif* NT processes was determined to be 55, 10, and 35%, respectively. Dagnino (1988) had shown that *cif* NT, measured using 10 µM adenosine, constituted 20% of total NT in L1210 cells at 25°C. These observations, combined with the apparent high-affinity (K_m = 20-40 µM) of the *cif* NT process (Dagnino, 1988; Vijayalakshmi and Belt, 1988), suggest that the Na⁺-dependent NT process of L1210 cells is a major route of nucleoside influx at physiological temperatures.

Results of the kinetic analyses revealed that the three functionally distinct NT processes of L1210/C2 cells were exhibited as two kinetically distinct NT activities. Although the K_m and V_{max} values for the low-affinity activity could not be graphically estimated, if a saturable transport process was responsible, an estimated K_m value in the mM range would not be unreasonable. The K_m and V_{max} values for the observed high-affinity transport activity were estimated to be 41 ± 10 µM and 8 ± 3 pmol/µl cell H₂O-sec. Chello *et al.* (1983) had previously observed high- and low-affinity adenosine transport activities in L1210 cells at 37°C and had assigned K_m values of 15.7 ± 1.5 and 191 ± 22 µM, respectively. The difference in K_m values observed for the high-affinity influx of adenosine and uridine are consistent with the general observation that the *es* (Plagemann *et al.*, 1988; Boleti, 1991), *cif* (Vijayalakshmi and Belt, 1988) and *ei* NT processes (Boleti, 1991) exhibit greater affinities for adenosine than uridine. While a specific NT process could not be assigned to either the high- and low-affinity activity in either the present study or that of Chello *et al.* (1983), it has been observed that K_m values for *es* and *cif* NT process are in the low µM range (Plagemann and Wohlheuter, 1988; Dagnino, 1988) whereas *ei* NT processes exhibit 3-5 fold higher K_m values (Boleti, 1991).

In this study, inhibition of N-linked glycosylation of newly synthesized plasma membrane proteins in L1210/C2 cells was accomplished by exposure to 2 μ M tunicamycin for a period of 24 hr. These exposure conditions were demonstrated to alter cellular metabolism and proliferation to a minimal extent. Inhibition of N-linked glycosylation induced the production of a population of plasma membrane NT proteins that altered the kinetic signature of NT processes in a complex manner. Results from kinetic analyses of uridine influx in tunicamycin-treated L1210/C2 cells indicated that the NT process(es) responsible for low-affinity transport exhibited lower K_m and V_{max} values as compared to untreated cells. While these results suggest that lack of N-linked glycosylation of the low-affinity NT process(es) is directly responsible for the change in kinetic parameters, a glycosylation-dependent change of NT protein from high- to low-affinity state cannot be excluded. The observation that erythrocyte equilibrative glucose transporter (GLUT1) lacking N-linked glycosylation exhibits lowered affinity would provide support the latter explanation (Asano *et al.*, 1991).

Total uridine influx in tunicamycin-treated L1210/C2 cells was observed to be 5-15% more sensitive to inhibition by ≥ 10 nM NBMPR than that of untreated cells, without a significant increase in IC_{50} values. Numbers of high-affinity NBMPR binding sites were similar in treated and untreated cells, suggesting that the increase in proportion of NBMPR-sensitive transport was not a result of increased abundance of *es* NT protein. This decrease in NBMPR-insensitive transport may be due to the observed decrease in *cif* NT process activity in tunicamycin-treated L1210/C2 cells. Whether this decreased activity was due to decreased expression of *cif* NT proteins at the plasma membrane or altered kinetic parameters of expressed non-glycosylated *cif* NT proteins could not be determined. Interestingly, the total transport activity mediated by the high-affinity process was identical in untreated and tunicamycin-treated cells, apparently a result of an increase in *es* NT activity in tunicamycin-treated L1210/C2 cells in conjunction with decreased *cif* NT process activity. This increased *es* NT activity was accompanied by the generation of a population of smaller (42-47 kDa), presumably deglycosylated, [^3H]NBMPR-photolabeled *es* NT protein that constituted approximately 15-20% of total photolabeled protein (Fig. II 17). It is tempting to speculate that L1210/C2 cells attempt to maintain a constant level of high-affinity NT activity by "activation" of a pre-existing NT process(es). Alternatively, the population of deglycosylated *es* NT protein may exhibit altered kinetic parameters.

A single study of the role of N-linked glycosylation in nucleoside transporter function has been reported by Olden *et al.* (1979). These authors provided evidence that uridine uptake, via a non-identified NT process(es), was reduced in tunicamycin-treated chicken fibroblasts. However, uridine uptake was measured over a 5-15 min interval, and the results likely reflected a combination of transport and metabolism of uridine and, as such, only suggested that glycosylation may be important in the function of NT processes. This study provides some circumstantial evidence that N-linked oligosaccharide of the *cif* NT proteins play a role in the functional expression of the transport process.

Endoglycosidase studies have demonstrated that N-linked oligosaccharide accounts for 11-30% of the total molecular weight of the [^3H]NBMPR-photolabeled *es* NT protein of rabbit, human and pig erythrocytes (Kwong *et al.*, 1993; Kwong *et al.*, 1992; Kwong *et al.*, 1986; Jarvis and Young, 1987; Good *et al.*, 1987), rat and guinea pig liver cells (Kwong *et al.*, 1993; Jarvis and Young, 1987), and human K562 erythroleukemia cells (Boleti, 1991). Kwong *et al.*, (1993) have shown that endo- β -galactosidase-mediated removal of terminal sugar residues from rat and guinea pig liver membranes did not interfere with the binding [^3H]NBMPR to the *es* NT protein. Apart from this observation, these studies did not address the type of N-linked oligosaccharide present nor any functional role that N-linked glycosylation may play in the transport process. Results from our study revealed that N-linked oligosaccharide is of tri- or tetraantennary

complex type and/or hybrid type and comprises 15-16% of the total molecular weight of the NBMPR-photolabeled *es* NT protein of L1210/C2 cells. It was also evident that the core oligosaccharide of the *es* NT protein was not necessary for high-affinity NBMPR binding.

A complication in this study was the simultaneous expression of *es*, *ei*, and *cif* NT processes in the L1210/C2 cell line. Recently, NT mutants of L1210 cells that express only *cif* NT (Crawford *et al.*, 1990) or *es* NT (Vijayalakshmi *et al.*, 1992) have been generated. The availability of these mutant cell types opens the possibility to define more precisely the role of N-linked glycosylation in the function of the *cif* and *es* NT proteins.

TABLE II 1

Kinetic Parameters for Uridine Zero-Trans Influx in L1210/C2 Cells Grown in the Absence or Presence of Tunicamycin

Velocities of uridine transport at 37°C were derived from time courses of [³H]uridine uptake in L1210/C2 cells that had been grown in the absence or presence of 2 μ M tunicamycin for 24 hr. Transport velocities of at least 10 concentrations, ranging from 1 - 550 μ M, was examined in three separate experiments conducted on different days. Kinetic parameters were derived by graphical analysis of Rosenthal plots (velocity versus velocity/concentration) according to Bylund (1980). The mean values ($\pm$ SD) for the three experiments are presented in bold type. *Values are from experiment presented in Figure II 13. (NA; not applicable).

Untreated Cells				Tunicamycin - Treated Cells			
Low - Affinity		High - Affinity		Low - Affinity		High - Affinity	
Km (μ M)	Vmax (μ M/sec)	Km (μ M)	Vmax (μ M/sec)	Km (μ M)	Vmax (μ M/sec)	Km (μ M)	Vmax (μ M/sec)
NA*	NA*	39*	10*	392*	186*	29*	8*
NA	NA	31	9	364	227	37	11
NA	NA	52	5	341	159	71	15
NA	NA	41 $\pm$ 10	8 $\pm$ 3	366 $\pm$ 26	191 $\pm$ 34	46 $\pm$ 21	11 $\pm$ 4

TABLE II 2

Binding Constants for Interaction of NBMPR with L1210/C2 Cells

Site-specific binding of [^3H]NBMPR to actively proliferating L1210/C2 cells at 20°C and 37°C was determined in either FTM or Na⁺-free buffer and the data were subjected to Rosenthal transformation. Binding constants (mean $\pm$ SD) were estimated after linear regression analysis of the binding data.

Binding Conditions	K _d (nM)	B _{max} (sites/cell $\times 10^{-5}$)
FTM* (20°)	0.13 $\pm$ 0.03	2.2 $\pm$ 0.6
FTM† (37°C)	0.24 $\pm$ 0.01	2.1 $\pm$ 0.2
Na ⁺ - free buffer (20°C)	0.07 $\pm$ 0.01	3.1 $\pm$ 0.2
<i>Cells treated with tunicamycin (2 μM, 24 hr)</i>		
FTM* (20°)	0.13 $\pm$ 0.02	2.0 $\pm$ 0.6
FTM† (37°C)	0.25 $\pm$ 0.01	2.2 $\pm$ 0.1

*Values are from 4-6 separate experiments, each with 3 determinations per condition. †Values are from a single experiment with three determinations per condition.

TABLE II 3

Exposure to Tunicamycin Differentially Alters the NBMPR-Sensitive and Na⁺-Dependent Influx Processes in L1210/C2 Cells

Transport velocities of 10 μ M uridine influx time courses presented in Figure II 11; as determined in FTM (total transport), FTM + 10 μ M NBMPR (NBMPR-insensitive transport), and Na⁺-free buffer + 10 μ M NBMPR (NBMPR-insensitive/Na⁺-independent transport) were calculated by linear regression analysis using the least squares method. Influx mediated by the NBMPR-sensitive and Na⁺-dependent processes were estimated by subtraction of transport velocities obtained in the presence of NBMPR and the absence of Na⁺.

	Transport Velocity (pmol/(μ l cell H ₂ O.sec))	
	- tunicamycin	+ 2 μ M tunicamycin
Assay Conditions:		
(a) Transport in FTM	2.32	2.10
(b) Transport in FTM + 10 μ M NBMPR	1.04	0.48
(c) Transport in Na ⁺ -free buffer + 10 μ M NBMPR	0.22	0.21
Transport Process:		
NBMPR-sensitive transport (a - b)	1.28 (55%)*	1.62 (77%)
Na ⁺ -dependent transport (b - c)	0.82 (35%)	0.27 (13%)
NBMPR-insensitive, Na ⁺ -independent transport (c)	0.22 (10%)	0.21 (10%)

*In parentheses are indicated the percent of total transport represented by each of the transport processes.

TABLE II 4

Prolonged Exposure to Low Concentrations of Tunicamycin Does Not Alter the Number of NBMPR-Binding Sites in Intact L1210/C2 Cells

L1210/C2 cells grown in the absence and presence of 0.8 μ M tunicamycin were harvested after 50 and 72 hr of exposure and resuspended at 0.5×10^6 – 4.0×10^6 cells/ml in FTM. Portions of cells (250 μ l) were incubated at RT for 15 min with an equal volume of 100 nM [^{3} H]NBMPR in the presence and absence of 10 μ M NBTGR. Cells were pelleted under oil by centrifugation and the radioactivity associated with the cell pellets was measured by liquid scintillation counting. Binding assays conducted in the presence of 10 μ M NBTGR were used to correct for non-specific binding and the specific NBMPR binding was converted from pmol/ 10^6 cells to the number of molecules (sites) per cell. Assays were conducted in triplicate and values presented (mean $\pm$ SD) are from 3 separate experiments.

	NBMPR Binding Sites (sites/cell $\times 10^{-5}$)	
	50 hr	72 hr
Untreated	1.34 ± 0.12	0.97 ± 0.12
Tunicamycin-Treated	1.39 ± 0.30	0.90 ± 0.18

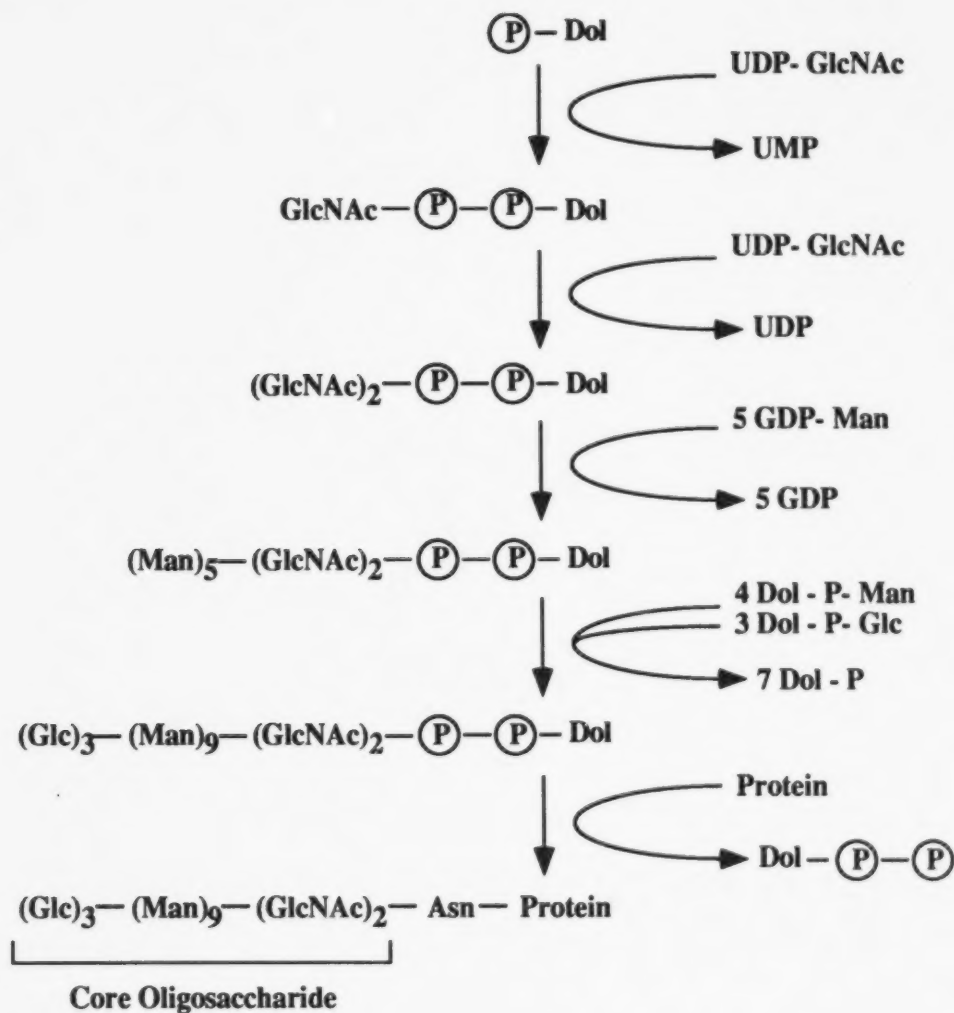


FIGURE II 1

Biosynthesis of Core N - Linked Oligosaccharide

Abbreviations: Dol, dolicol; Glc, glucose; GlcNAc, N - acetylglucosamine; Man, mannose; P, phosphate)

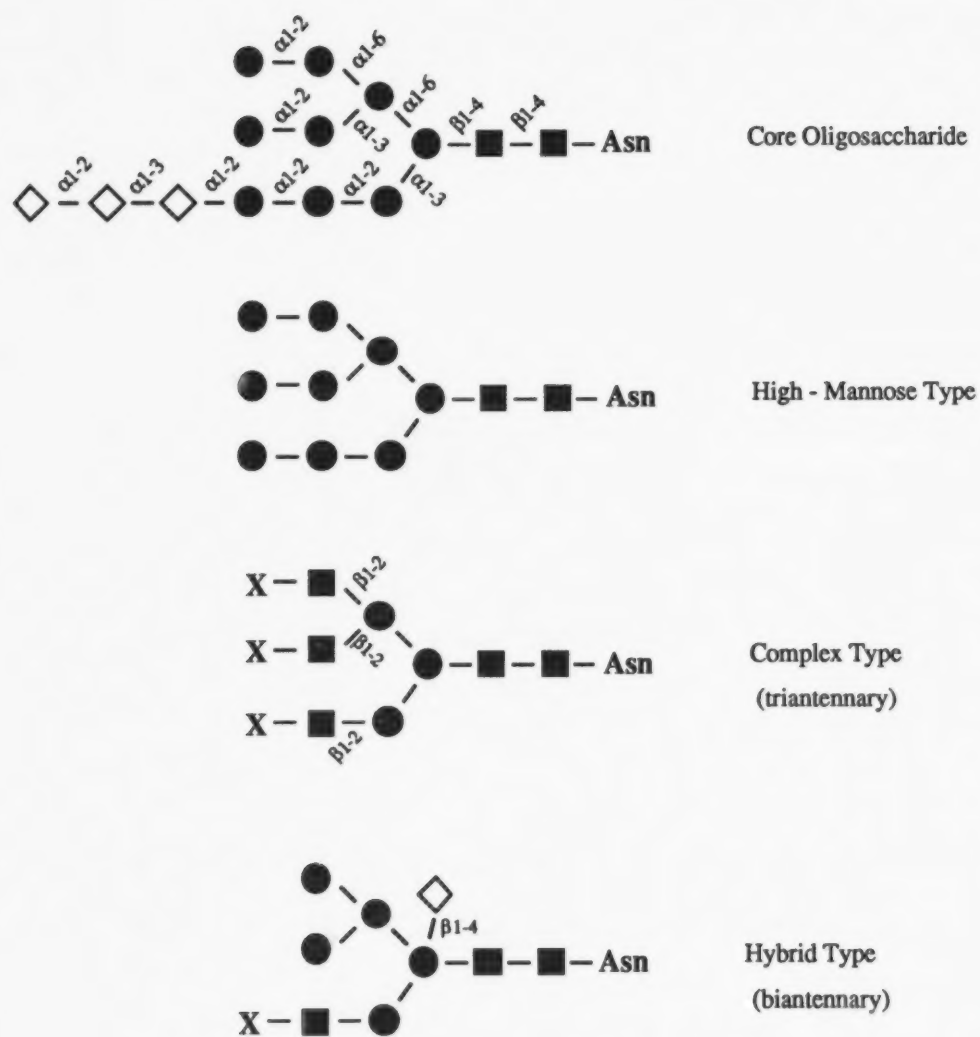


FIGURE II 2

A Schematic Illustration of the Types of N - Linked Oligosaccharide Chain

Symbols: $\blacksquare$, N - acetylglucosamine; $\bullet$, mannose; $\diamond$, glucose; X, variable oligosaccharide.
Bond linkages are indicated between symbols.

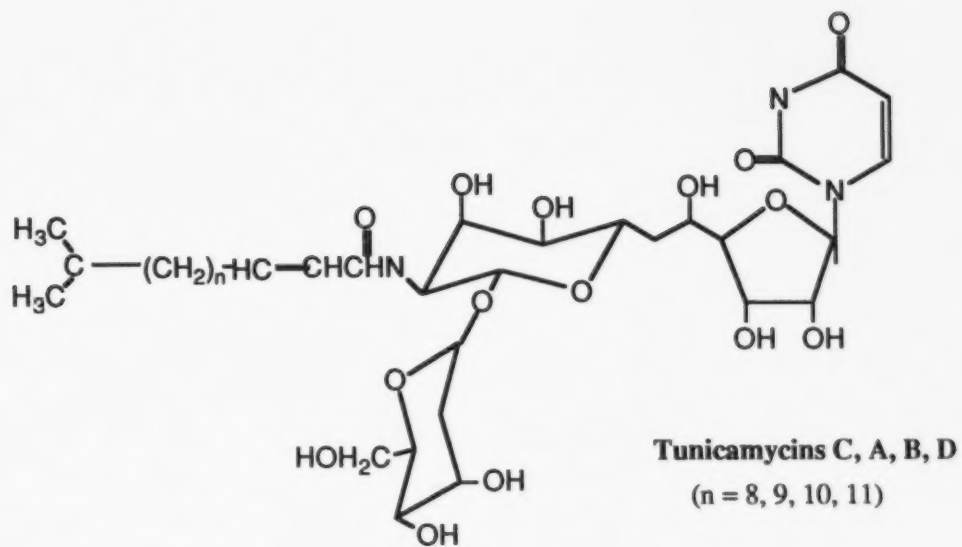


FIGURE II 3
Structure of Tunicamycin

FIGURE II 4

Tunicamycin Alters the Proliferation of L1210/C2 cells in a Concentration-Dependent Manner

Actively proliferating L1210/C2 cells were harvested by centrifugation (150 x g, 5 min) and resuspended (1.0×10^5 cells/ml) in 10 ml fresh growth medium that contained 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 μM tunicamycin. Cell concentrations were determined after 24 (●), 48 (▲), and 72 (■) hr incubation. Proliferative rates (number of cell doublings/24 hr) in the presence of tunicamycin are expressed as percentages of rates observed in the absence of tunicamycin (mean $\pm$ SD for 3 determinations). Untreated cells exhibited a population doubling time of 14 ± 1 hr (mean $\pm$ SD for 3 determinations). Error bars are not shown where SD values were smaller than size of data symbols. The IC_{50} values for the 72, 48, and 24 hr exposures were 1.3, 1.6, and >3.0 μM respectively.

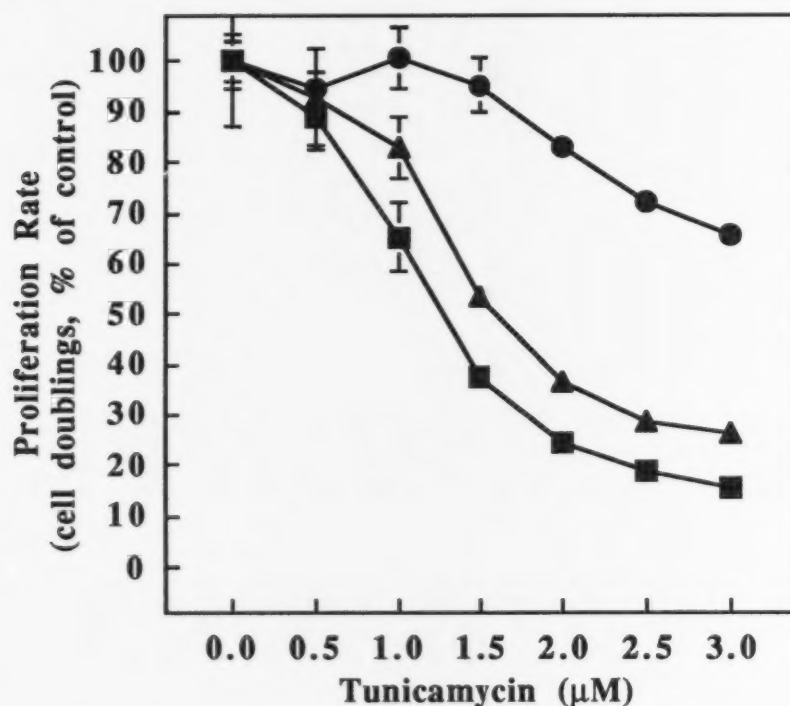


FIGURE II 5

Mannose Incorporation by L1210/C2 Cells is Preferentially Inhibited During Exposure to Tunicamycin

Replicate cultures of actively proliferating L1210/C2 cells (2×10^5 cells/ml) were grown in Fishers medium containing $0.25 \mu\text{Ci/ml}$ of L- ^3H leucine, $0.25 \mu\text{Ci/ml}$ of D- ^3H thymidine, or $1.25 \mu\text{Ci/ml}$ of D- ^3H mannose in the presence of 0, 1.0, 2.0, and 3.0 μM tunicamycin. Cells were harvested after 24 hr of incubation by centrifugation and acid - insoluble precipitates were prepared. The radioactivity and protein content of ^3H thymidine (■), ^3H leucine (▲), and ^3H mannose (●)-labeled samples were determined and ^3H -labeled precursor incorporation (cpm/mg protein) in the presence of tunicamycin was calculated as percentage of incorporation in the absence of tunicamycin. Data (mean $\pm$ SD) from 4 separate experiments are shown.

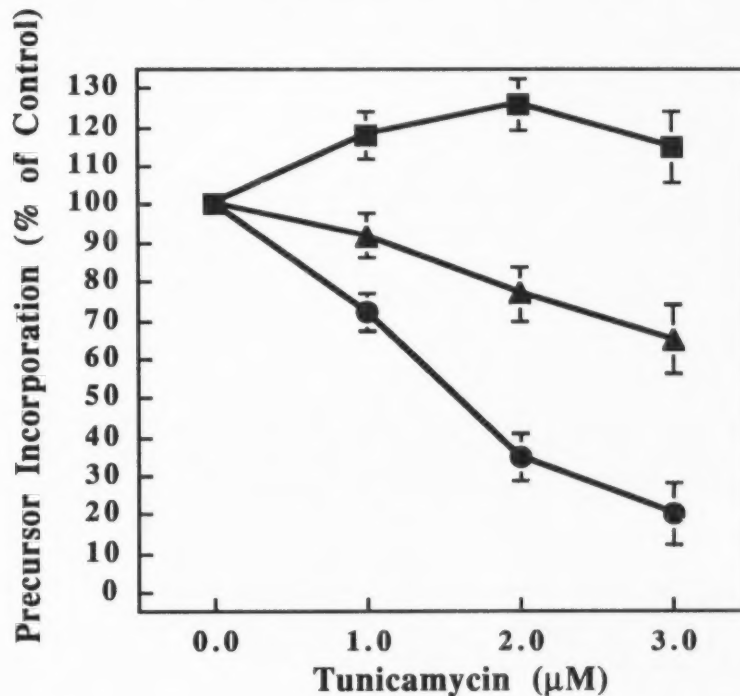


FIGURE II 6

Exposure to Tunicamycin Inhibits the Incorporation of [^3H]Mannose into Newly Synthesized Plasma Membrane Glycoproteins

Suspensions of L1210/C2 cells (1.5×10^5 cells/ml) in Fishers medium were pre-incubated for 2 hr in the absence (●) or presence (○) of 2 μM tunicamycin, then [^3H]mannose was added (final concentration: 0.4 $\mu\text{Ci/ml}$, 60 pM) and cells were grown for an additional 24 hr. Cells were harvested by centrifugation and fractions enriched in plasma membrane were prepared by Percoll gradient separation. The plasma membrane fractions (50 μg protein) were subjected to electrophoretic separation on a 12% SDS-polyacrylamide gel and lanes containing ^3H -labeled samples were cut into 2 mm slices. Gel slices were solubilized and radioactivity was determined by liquid scintillation counting. The positions of migration of molecular weight standards are shown (SG, stacking gel interface; TD, position of tracking dye).

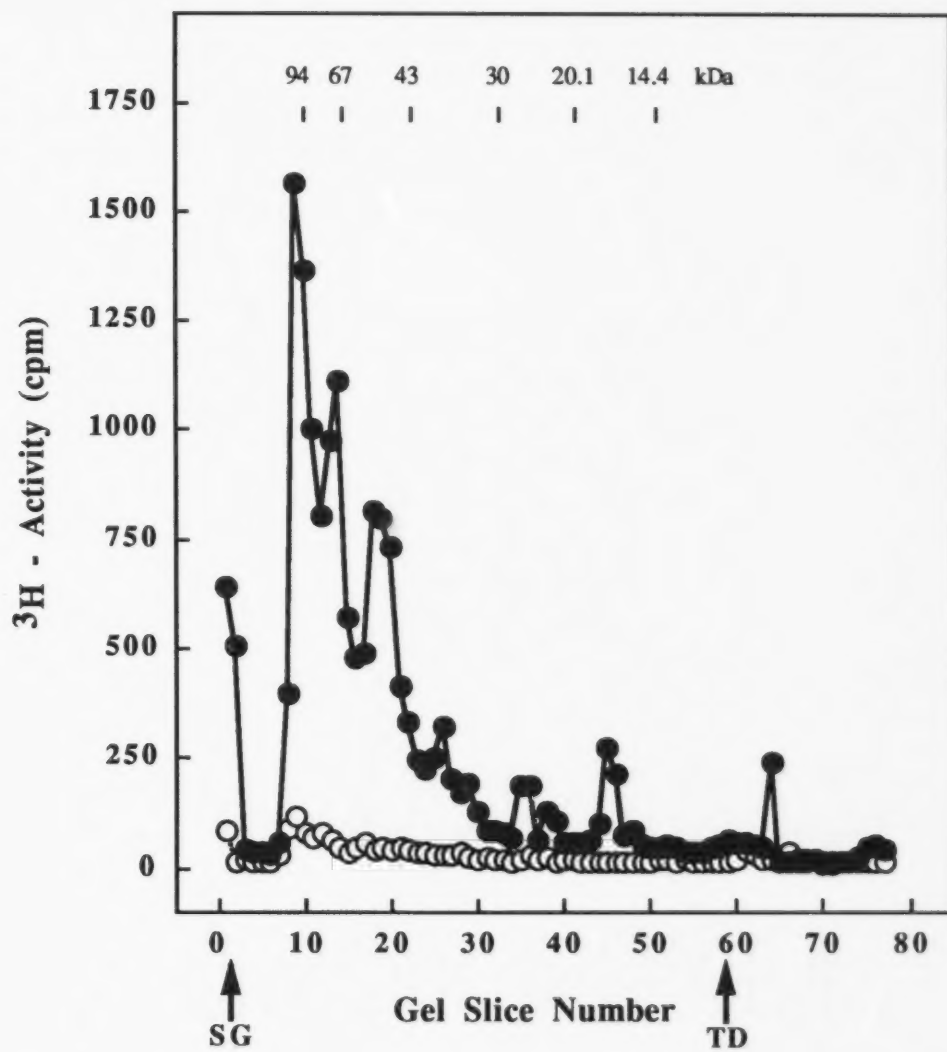


FIGURE II 7

Turnover of [^3H]Mannose-Labeled Glycoproteins in the Plasma Membrane is Not Altered During Exposure to Tunicamycin

A culture of actively proliferating L1210/C2 cells was diluted to 1.0×10^5 cells/ml and grown in the presence of [^3H]mannose (final concentration: $0.4 \mu\text{Ci/ml}$, 57 pM) for 45 hr. The cells were collected by centrifugation, washed twice with PBS, and resuspended into two equal volumes at 1.0×10^5 cells/ml in fresh [^3H]mannose-free Fishers medium. The cell suspensions were grown an additional 24 hr in the absence (●) or presence (□) of $2 \mu\text{M}$ tunicamycin. Fractions enriched for plasma membranes were prepared by the Percoll gradient method from cells harvested by centrifugation at 0, 12, and 24 hr intervals after the time of addition of tunicamycin. Radioactivity of triplicate samples from each time point was determined by liquid scintillation counting. Data (mean $\pm$ SD) were calculated assuming $2.2 \times 10^3 \text{ dpm}/\mu\text{Ci}$ and expressed as pmol mannose/ μg protein. Error bars are not shown were SD values were smaller than the size of the data symbols.

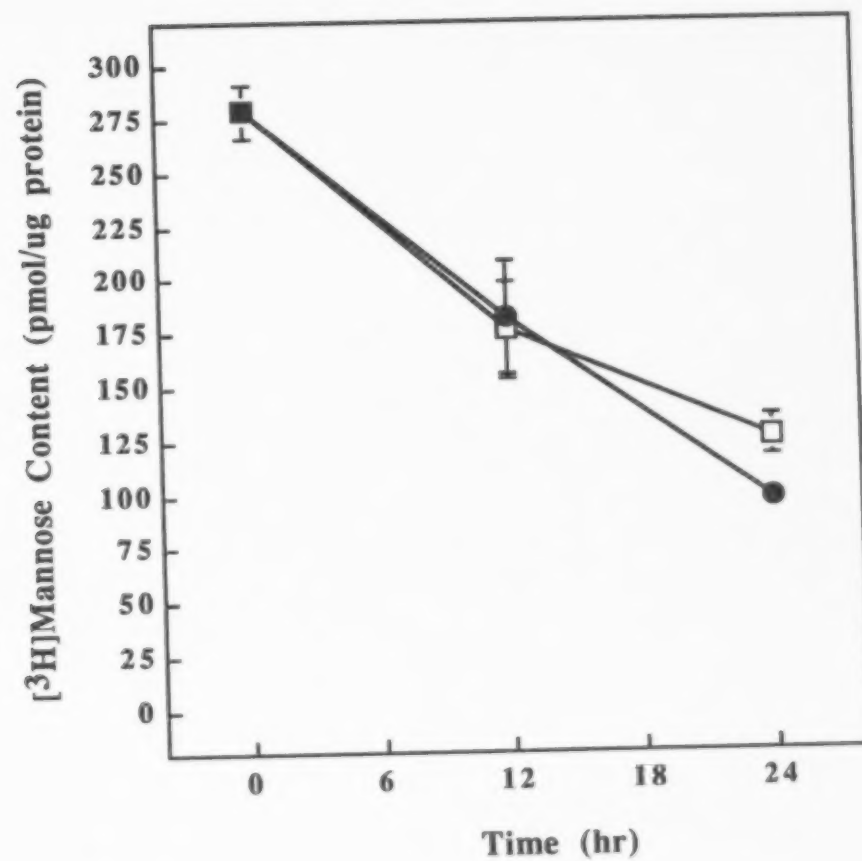


FIGURE II 8

Addition of Ice-Cold Dilazep Instantaneously Inhibits Uridine Flux at 37°C in L1210/C2 Cells

Portions of L1210/C2 cell suspensions (100 μ l, 2×10^7 cells/ml) were incubated with [^{3} H]uridine (100 μ l, 11 and 382 μ M) at 37°C over 0-10 sec and then subjected to centrifugation through oil ("oil-stop") at 2 sec intervals (●) during the incubation, as described in Materials and Methods. In some cases, 200 μ l of ice-cold (4°C) dilazep (□) or 37°C dilazep (Δ) was added after 6 sec of incubation and cells were (i) immediately centrifuged through oil or (ii) left to incubate an additional 4 or 12 sec prior to initiation of centrifugation. Data for oil stop assays were means $\pm$ SD for three determinations and for dilazep stop assays were arithmetic means of two determinations. Error bars are not shown were SD values were smaller than the size of the data symbols.

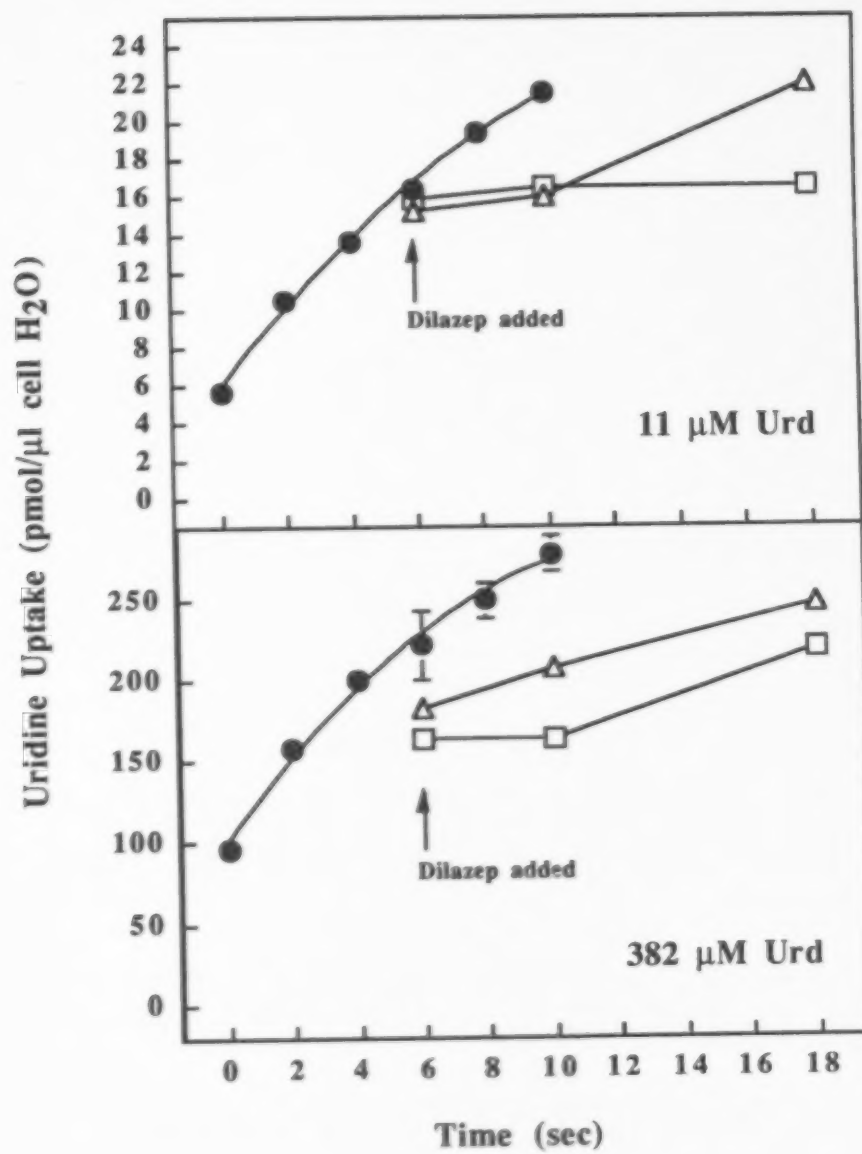


FIGURE II 9

Uridine is Rapidly Metabolized After Transport Into L1210/C2 cells at 37°C

Uptake intervals were started by addition of portions of L1210/C2 cell suspensions (100 μ l, 2×10^7 cells/ml) to microcentrifuge tubes (1.5 ml) that had been layered, from bottom to top, with 100 μ l of 4% (v/v) PCA, 200 μ l of transport oil, and 100 μ l of D- ^{3} H]uridine (8 or 378 μ M) in FTM. Uptake (at 37°C) was stopped at 5 or 10 sec by the addition of 200 μ l ice - cold dilazep solution and cells were immediately pelleted into the PCA layer by centrifugation (9000 x g, 10 sec). Zero-time samples were obtained by simultaneous addition of cells and dilazep solution to microfuge tubes followed by centrifugation. The upper aqueous and oil layers were removed and the PCA fraction was neutralized with Alamine 336 (in Freon-TF) and subjected to centrifugation (9000 x g, 4 min). The resulting aqueous phase was subjected to TLC in isopropanol/saturated ammonium sulfate/H₂O (2:79:19 v/v). The radioactivity migrating at positions corresponding to that of uracil (open bars), uridine (solid bars), or 5'-phosphorylated uridine metabolites (UMP, UDP, UTP; diagonally-hatched bars) was determined by liquid scintillation counting. Data are single time point assays expressed as a percentage of total radioactivity subjected to TLC and are results from a single representative experiment.

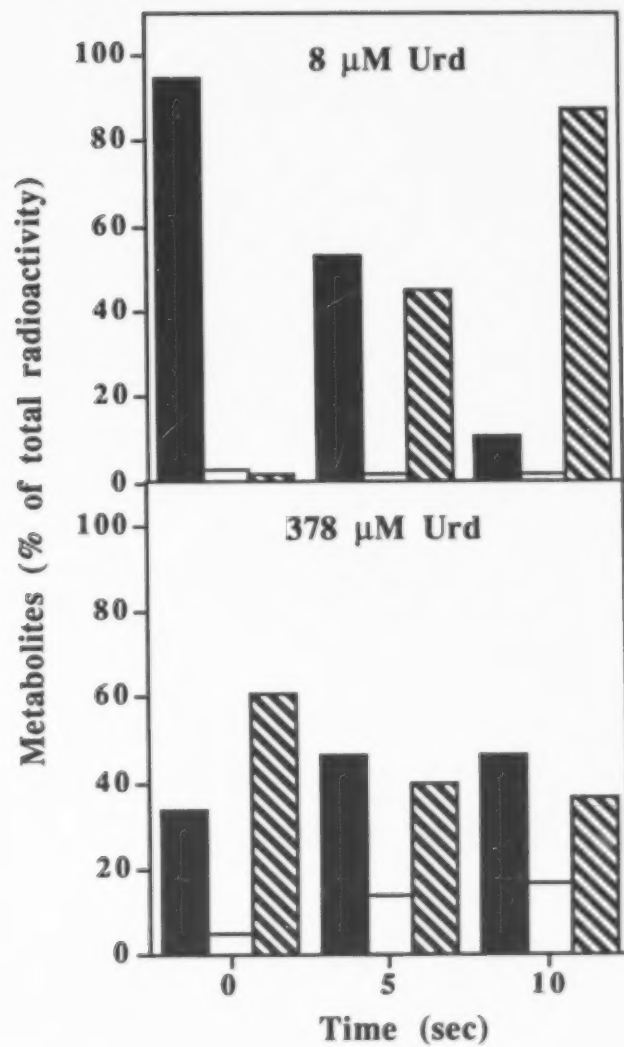


FIGURE II 10

Representative Time Courses for Zero-Trans Influx of Uridine in L1210/C2 Cells at 37°C

Actively proliferating L1210/C2 cells suspended in FTM (100 μ l, 2×10^7 cells/ml) were used to measure influx of graded concentrations of [^{3} H]uridine at 2 sec intervals over a 0 - 6 sec period as described in Materials and Methods, with the following modifications. Cells were prepared from 4 consecutive harvests of a single culture, each of which was used to measure influx at 3-4 randomly selected concentrations. Uptake was terminated by the addition of 200 μ l of ice-cold dilazep (200 μ M) in FTM followed by immediate centrifugation. The zero-time values were obtained by the simultaneous addition of cells and dilazep solution and immediate centrifugation. For clarity, the time courses for 1, 6, 10, and 15 μ M are shown with an expanded scale in the insert. Values are the means of 2 or 3 assays, each of which was conducted separately.

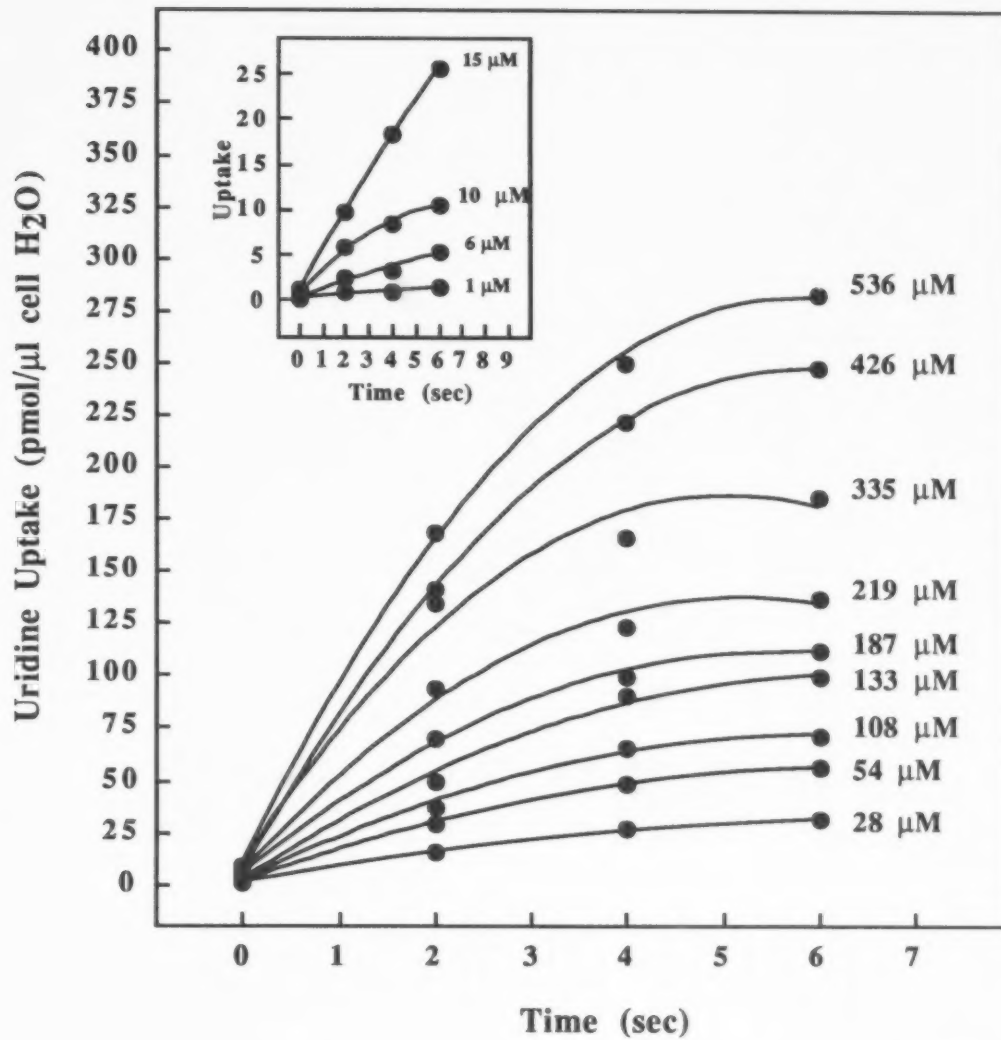


FIGURE II 11

Representative Time Courses for Zero-*Trans* Influx of Uridine in Tunicamycin - Treated L1210/C2 Cells at 37°C

L1210/C2 cells were grown in the presence of 2 μM tunicamycin for 24 hr, then centrifuged and suspended in FTM (2×10^7 cells/ml) for use in uptake assays. Portions (100 μl) of cell suspension were used to measure influx of graded concentrations of [^3H]uridine at 1 sec intervals over a 0-3 sec period, as described in Fig. II 10. For clarity, the time courses for 1, 3, 6, 13, and 21 μM are shown with an expanded scale in the insert. Values are means of 2 or 3 assays, each of which was conducted separately.

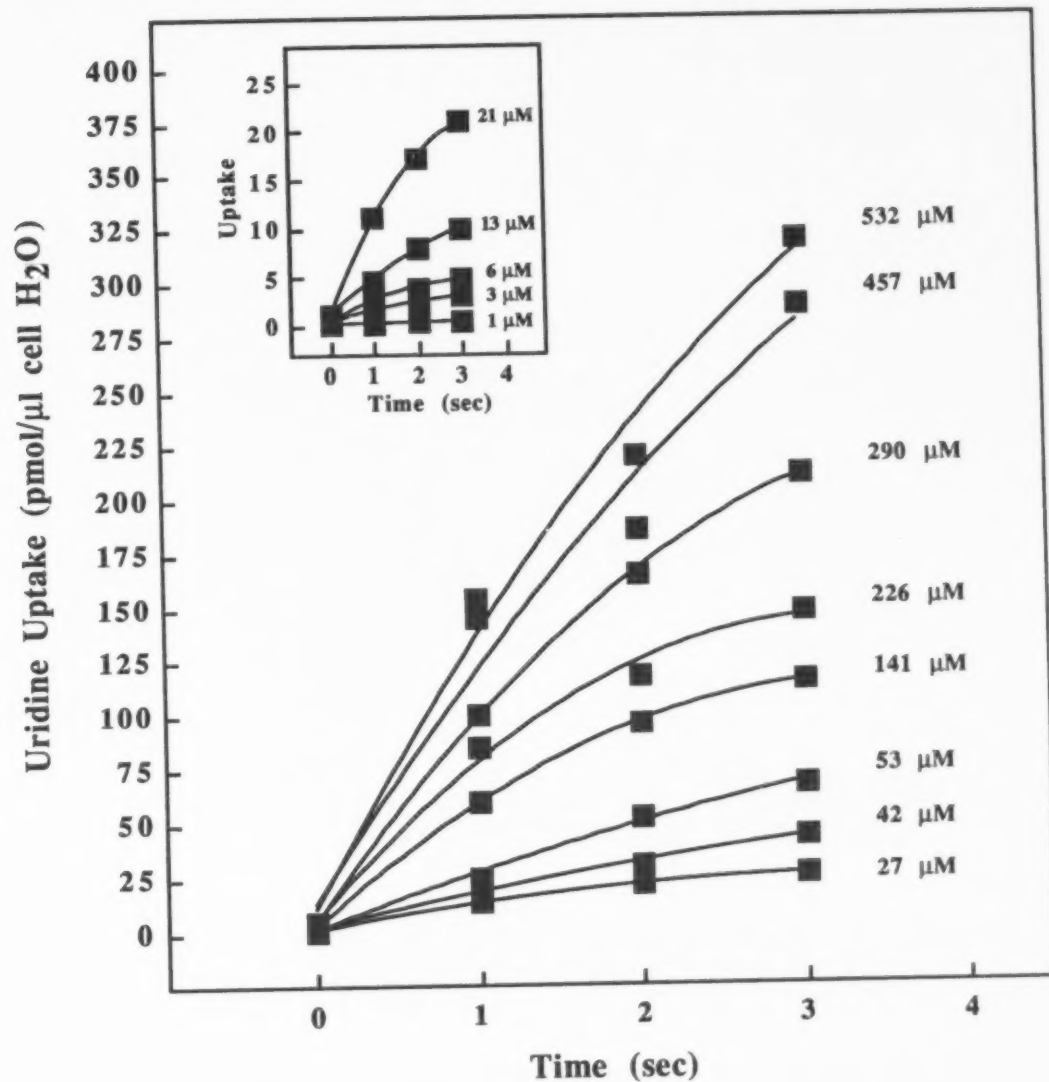


FIGURE II 12

L1210/C2 Cells Exposed to Tunicamycin Exhibit Altered Initial Rates of Uridine Transport at 37°C

Initial transport velocities at 37°C were determined using cells grown in the absence (●) or presence (□) of 2 μ M tunicamycin for 24 hr. Time courses of zero-*trans* [^{3} H]uridine influx for a concentration range of 0.5 to 420 μ M uridine were obtained as described in Figs. II 10 and 11 and velocities were calculated by either linear regression or polynomial regression using the least squares method, as described in Materials and Methods. The results of one of three separate experiments, each conducted on separate days and giving rise to similar results, are presented. The values obtained for untreated and tunicamycin-treated cells, respectively, for (i) cellular H₂O content (mean $\pm$ SD for 4 determinations) were 0.56 ± 0.02 and 0.49 ± 0.03 μ l/10⁶ cells and for (ii) sucrose space (range) were 5.0-5.8 and 5.5-6.8% of the total pellet volume.

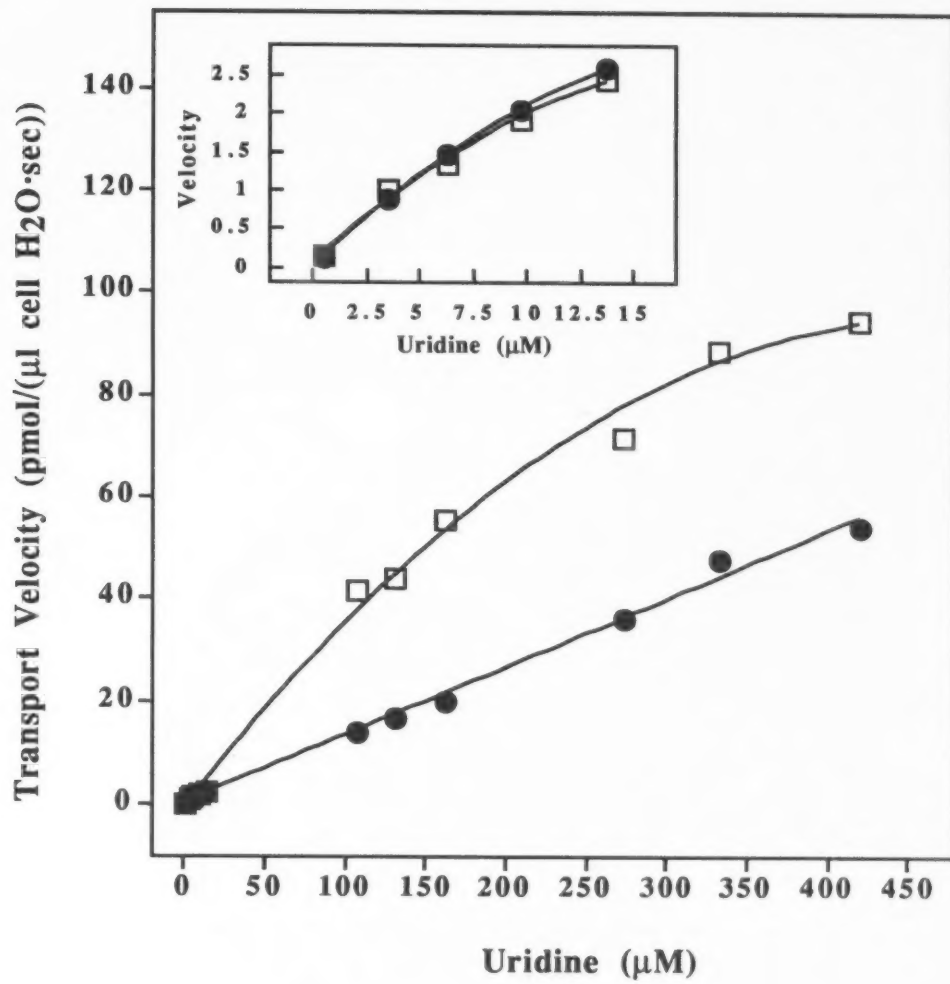


FIGURE II 13

L1210/C2 Cells Express Two Kinetically Distinguishable Components of Uridine Transport at 37°C

Results from the experiment of Figure II 12 are presented as Rosenthal plots (velocity/concentration versus velocity) for cells grown in the absence (●) or presence (□) of 2 μ M tunicamycin for 24 hr. K_m (-1/slope) and V_{max} (x axis-intercept) values for the low- and high-affinity transport activities were estimated by linear regression analysis of the data by the least squares method.

Panel A: Untreated L1210/C2 cells. K_m and V_{max} values for high-affinity transport were 39 μ M and 10 pmol/ μ l cell H₂O-sec, respectively. Kinetic values of low-affinity transport in untreated cells could not be estimated due to the apparent non-saturable nature of the process.

Panel B: Tunicamycin-treated L1210/C2 cells. K_m and V_{max} values for high-affinity and low-affinity transport were 29 and 392 μ M, and 8 and 186 pmol/ μ l cell H₂O-sec, respectively.

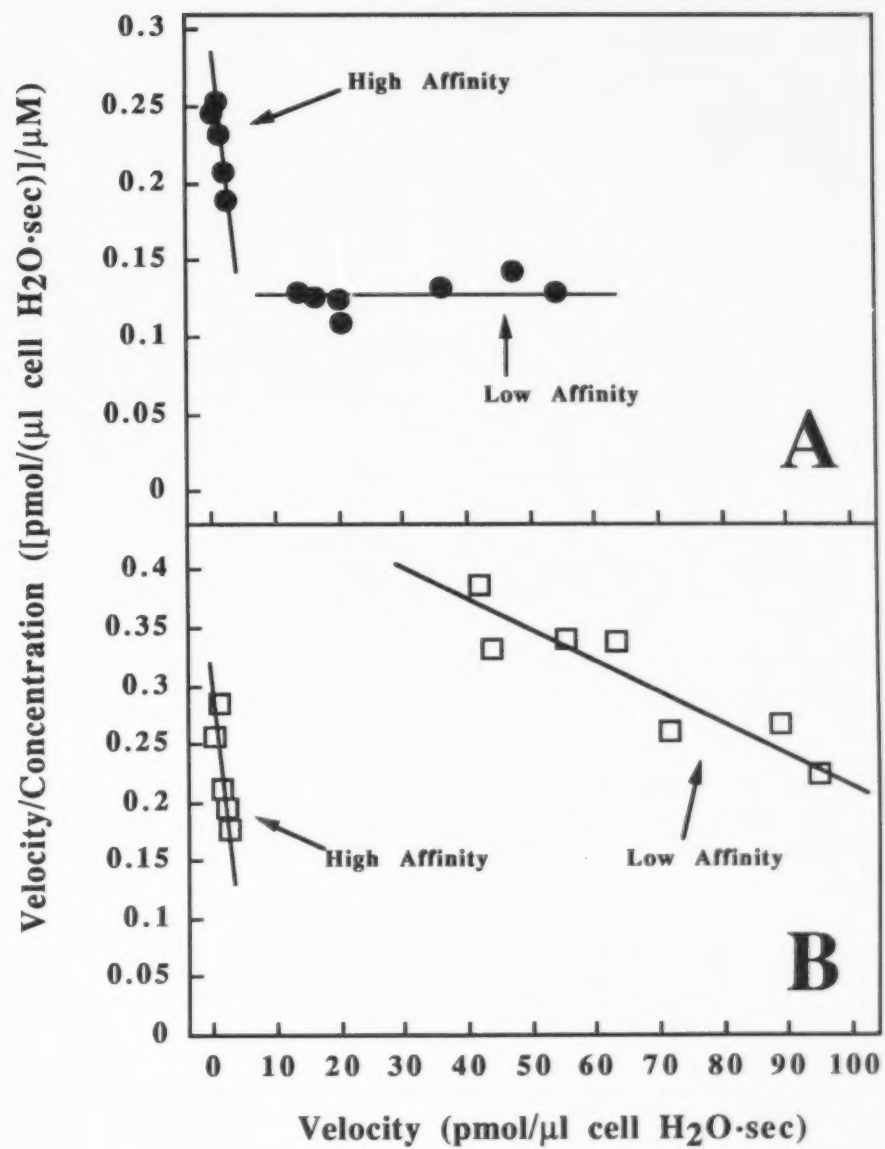


FIGURE II 14

Concentration Dependence of NBMPR Inhibition of Uridine Transport at 37°C Appears to be Slightly Altered by Exposure to Tunicamycin

Suspensions of cells (10^5 cells/ml) in FTM were prepared from cultures grown for 24 hr in the absence (●) or presence (□) of 2 μ M tunicamycin and exposed to graded concentrations (0-10 μ M) of NBMPR for 30 min at 37°C. The cells were harvested and resuspended (2×10^7 cells/ml) in FTM that contained the same concentrations of NBMPR. Measurements of influx of 1 and 300 μ M uridine were performed at 37°C using the procedure described in Fig. II 10, with the following modification. The 100 μ l portion of [^{3} H]uridine in FTM used in the assay also contained the same concentration of NBMPR as that of the cell suspension with which it was combined. Initial velocities of uridine transport were calculated from time courses of uptake by the least squares method. Transport velocities obtained at 1 μ M and 300 μ M uridine in the presence of NBMPR are expressed as the percentage of the values obtained in the absence of NBMPR.

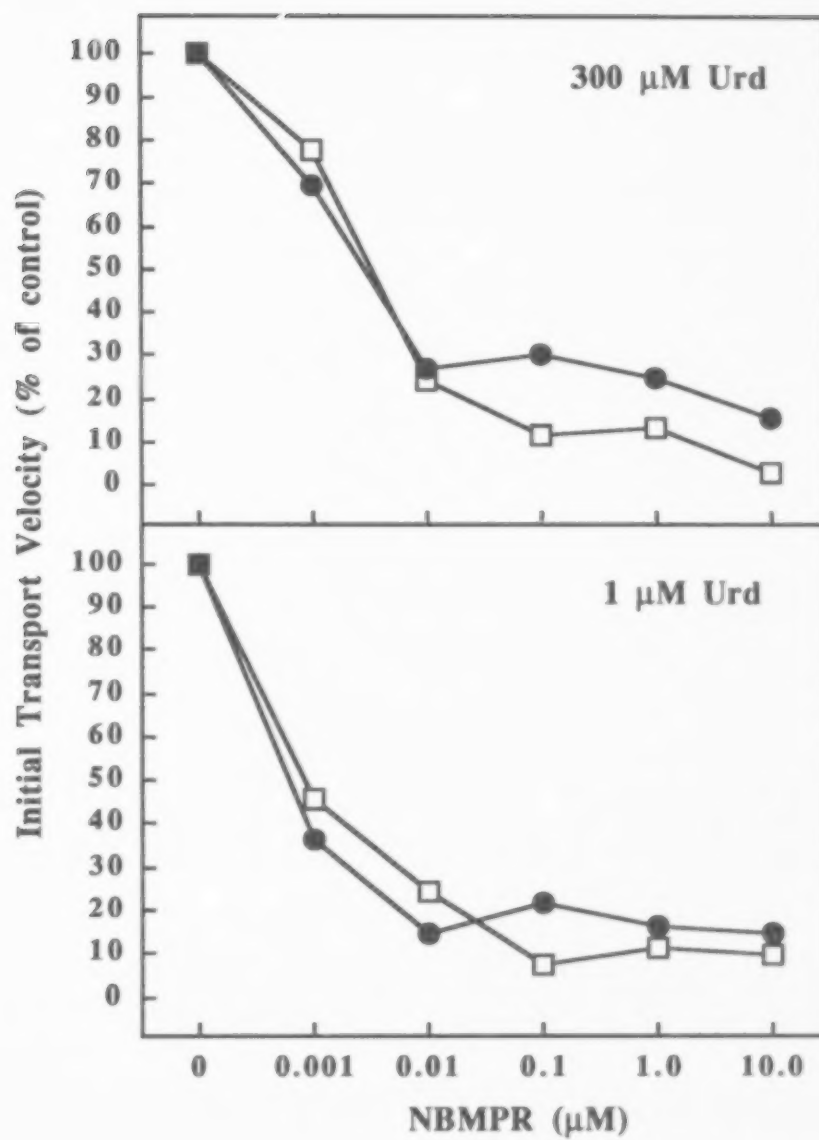


FIGURE II 15

Exposure to Tunicamycin Does Not Affect the Equilibrium Binding of NBMPR With L1210/C2 Cells

Panel A: Equilibrium binding of [^{3} H]NBMPR. Cells that had been grown for 24 hr in the presence ($\Delta, \blacktriangle$) and absence ($\bullet, \circ$) of 2 μ M tunicamycin were harvested and incubated at 37°C for 30 min in FTM containing graded concentrations of [^{3} H]NBMPR (0.1-8 nM) alone ($\blacktriangle, \bullet$) or also containing 10 μ M NBTGR ($\Delta, \circ$). For each concentration of NBMPR, the cell-associated and actual free concentrations of [^{3} H]NBMPR were determined as described in Materials and Methods. Values for total bound NBMPR ($\blacktriangle, \bullet$) and non-specifically bound NBMPR ($\Delta, \circ$) are means of three determinations. SD values (not shown) ranged between 0.3-4.5% of the mean values.

Panel B: Rosenthal transformation. Specifically-bound NBMPR for untreated ($\bullet$) and tunicamycin-treated cells (Δ) was calculated by subtracting values for non-specific binding from those for total binding. Linear regression analysis was used to estimate values for K_d (-1/slope) and B_{max} (x-intercept) and the B_{max} value was converted from pmol/ 10^7 cells to the number of molecules (sites) per cell. For tunicamycin-treated and untreated cells, the binding constants obtained were, respectively: K_d , 0.25 ± 0.01 and 0.24 ± 0.01 nM; B_{max} , $2.2 \times 10^5 \pm 0.1 \times 10^5$ and $2.1 \times 10^5 \pm 0.2 \times 10^5$ sites/cell. These values are included in the data presented in Table II 2.

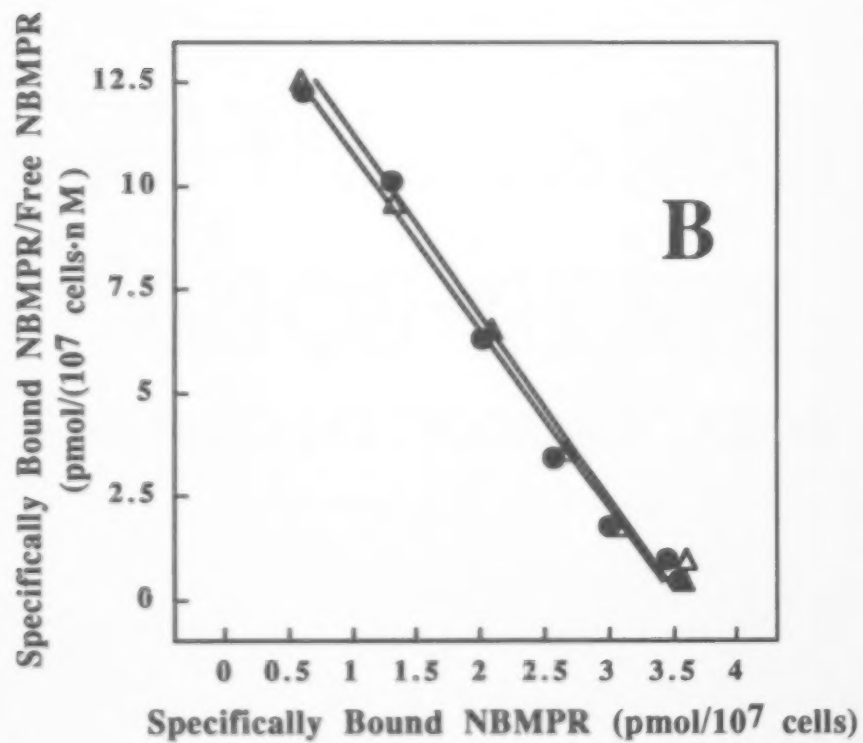
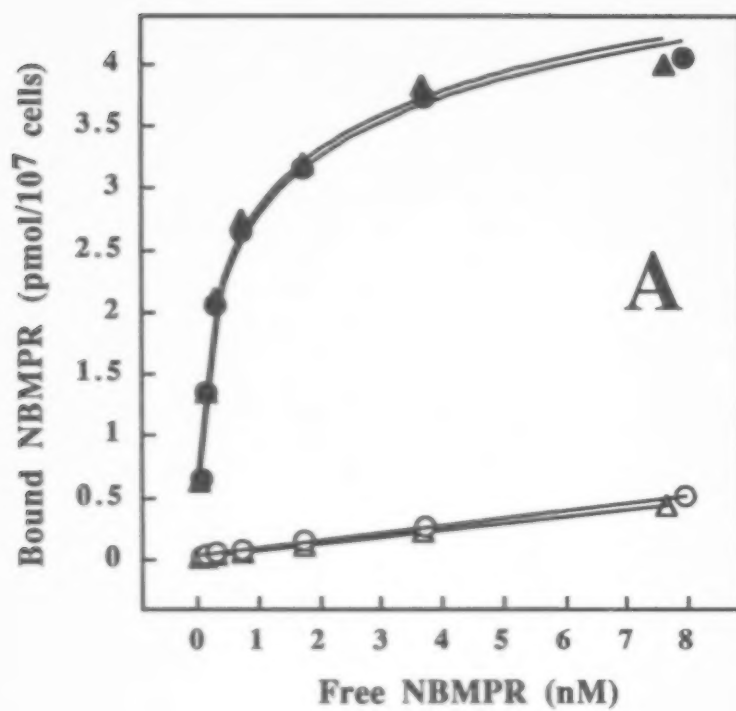


FIGURE II 16

Tunicamycin Exposure Alters the Contribution of Three Functionally Distinct Processes To Total *Zero-Trans* Influx of Uridine at 37°C

Actively proliferating cells, grown for 24 hr in the absence (●,▲,■) or presence (○,△,□) of 2 μM tunicamycin, were incubated (1.5×10^6 cells/ml, 10 min, 37°C) in FTM or FTM containing 10 μM NBMPR. The cell suspensions were harvested (1.5×10^7 cells/ml) for assay of initial rates of uptake of 10 μM [^3H]uridine at 37°C, as described in Figure II 7. Time courses are presented for cells incubated and assayed in FTM (●,○); cells incubated and assayed in FTM containing 10 μM NBMPR (▲,△); and cells incubated in FTM containing 10 μM NBMPR and assayed in Na^+ -free buffer containing 10 μM NBMPR (■,□). Data shown are mean $\pm$ SD for three determinations per time point. Error bars are not shown where SD values were smaller than the size of the data symbols.

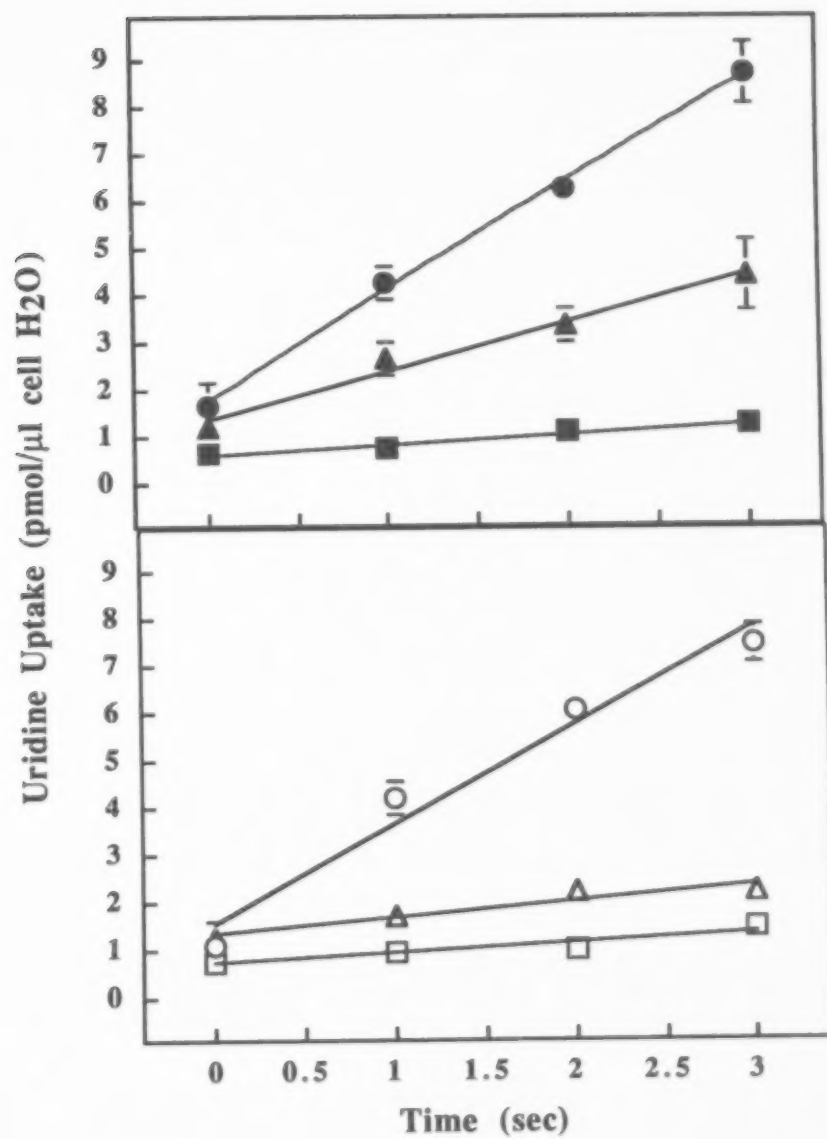


FIGURE II 17

Exposure to Tunicamycin Generates a Lower Molecular Weight Species of [^3H]NBMPR-Labeled Protein in the Plasma Membranes of L1210/C2 Cells

Plasma membrane fractions were prepared from L1210/C2 cells (1×10^9 cells) grown for 24 hr in the absence (●) or presence (□) of 2 μM tunicamycin and subjected to UV-photolabeling with 100 nM [^3H]NBMPR as described in Materials and Methods. Non-specific labeling with [^3H]NBMPR was determined by including 10 μM NBTGR (○) in the photolabeling reaction. Equal quantities (75 μg protein) of irradiated membranes were solubilized under reducing conditions and subjected to electrophoresis on a 12% SDS-polyacrylamide gel. Migration of photolabeled proteins was determined by slicing lanes into 2 mm slices and estimating the ^3H -content in each slice by liquid scintillation counting. The positions of molecular weight standards were determined by staining with Coomassie blue and are indicated at the top of the figure. The position of the stacking gel interface (SG) is indicated.

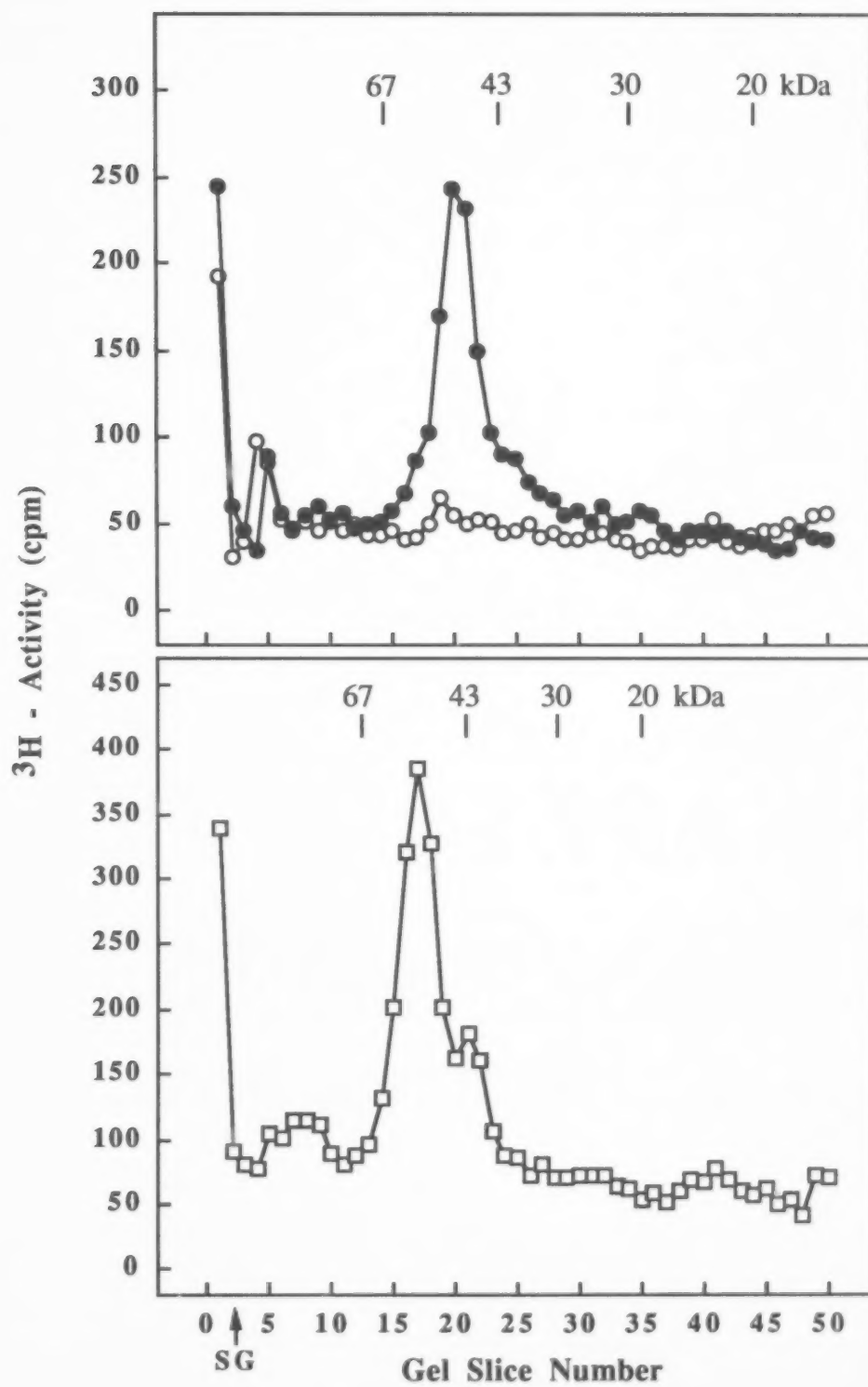


FIGURE II 18

Prolonged Exposure to Low Concentrations of Tunicamycin Converts the Plasma Membrane [³H]NBMPR-Labeled Protein to a Lower Molecular Weight Species

L1210/C2 cells, initially cultured at 0.8×10^5 cells/ml, were grown in medium containing $0.8 \mu\text{M}$ tunicamycin for a 72 hr period. Plasma membrane fractions were prepared from portions of cultured removed after 0, 36, 50, and 72 hrs of exposure using the Percoll gradient centrifugation procedure described in Materials and Methods. The membrane fractions were simultaneously subjected to UV-photolabelling with 100 nM [³H]NBMPR. Equal quantities ($45 \mu\text{g}$ protein) of irradiated membranes were solubilized and subjected to electrophoresis on a 12% polyacrylamide gel. Migration of photolabeled proteins was determined by slicing lanes into 2 mm slices and counting the ³H-content of each slice. The positions of molecular weight standards were determined by staining with Coomassie blue and are indicated in each panel.

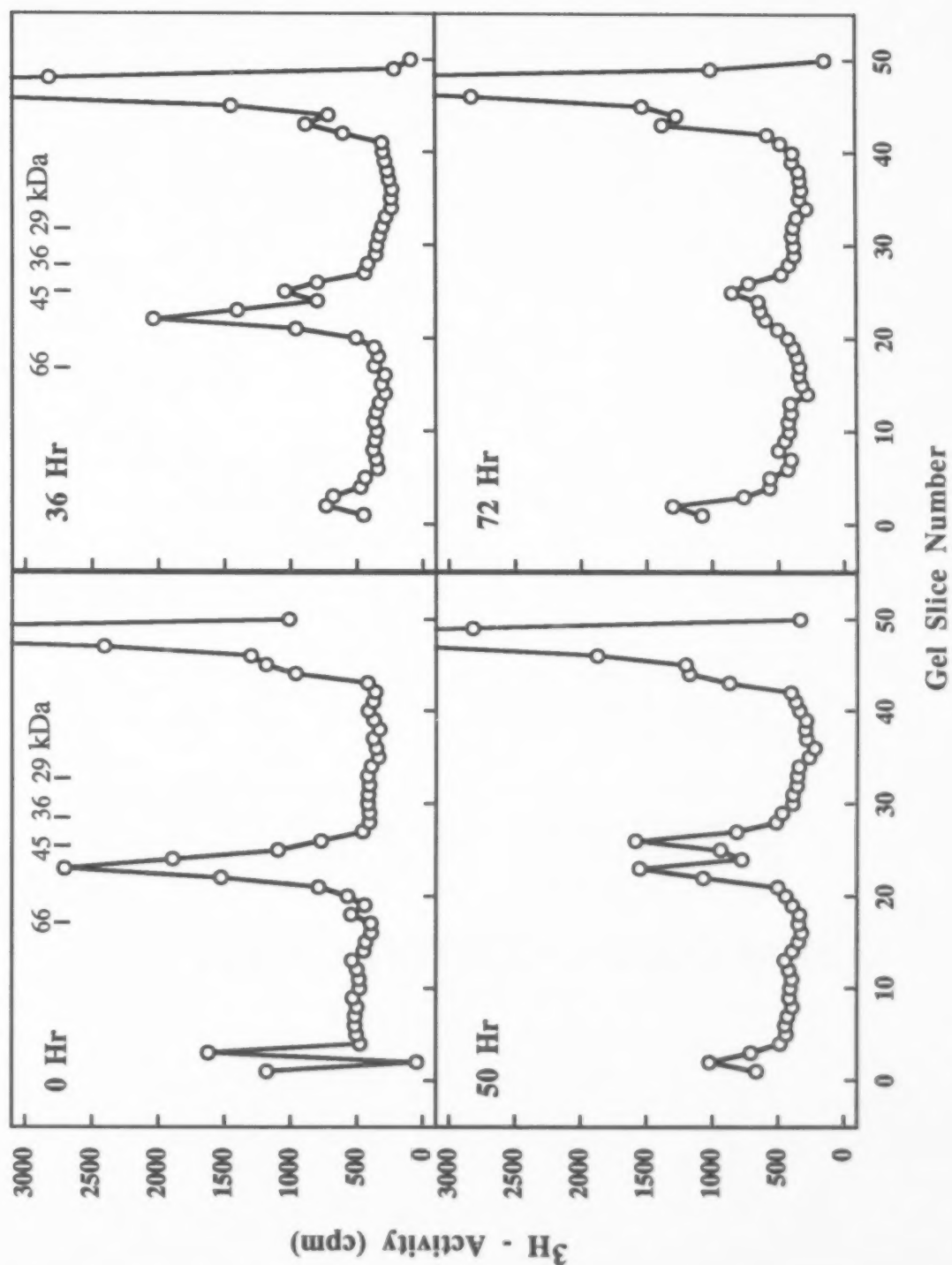


FIGURE II 19

The L1210/C2 Plasma Membrane [^3H]NBMPR-Labeled Protein is Insensitive to Digestion by O-Glycanase

Plasma membrane fractions were prepared from actively proliferating L1210/C2 cells and subjected to UV-photolabeling with 100 nM [^3H]NBMPR. Irradiated membranes were washed with ice-cold 20 mM Tris-HCl (pH 7.2) and collected by centrifugation. Replicate samples of photolabeled membranes were utilized in the experiments shown in Figures II 19, 20 and 21. Portions of photolabeled membranes ($\approx 250 \mu\text{g}$ protein) were resuspended in 100 mM CH_3COONa /19 mM KH_2PO_4 (pH 6.5) and sequentially incubated in the absence (bottom panel) or presence of 0.5 U of neuraminidase and 5 mU of O-Glycanase (top panel), as described in Materials and Methods. The reaction mixtures were solubilized under reducing conditions and subjected to electrophoresis on a 12% SDS-polyacrylamide gel. Migration of photolabeled proteins was determined by slicing lanes into 2 mm slices and estimating the ^3H -content in each slice by liquid scintillation counting. The positions of molecular weight standards were determined by staining with Coomassie blue and are indicated at the top of the figure.

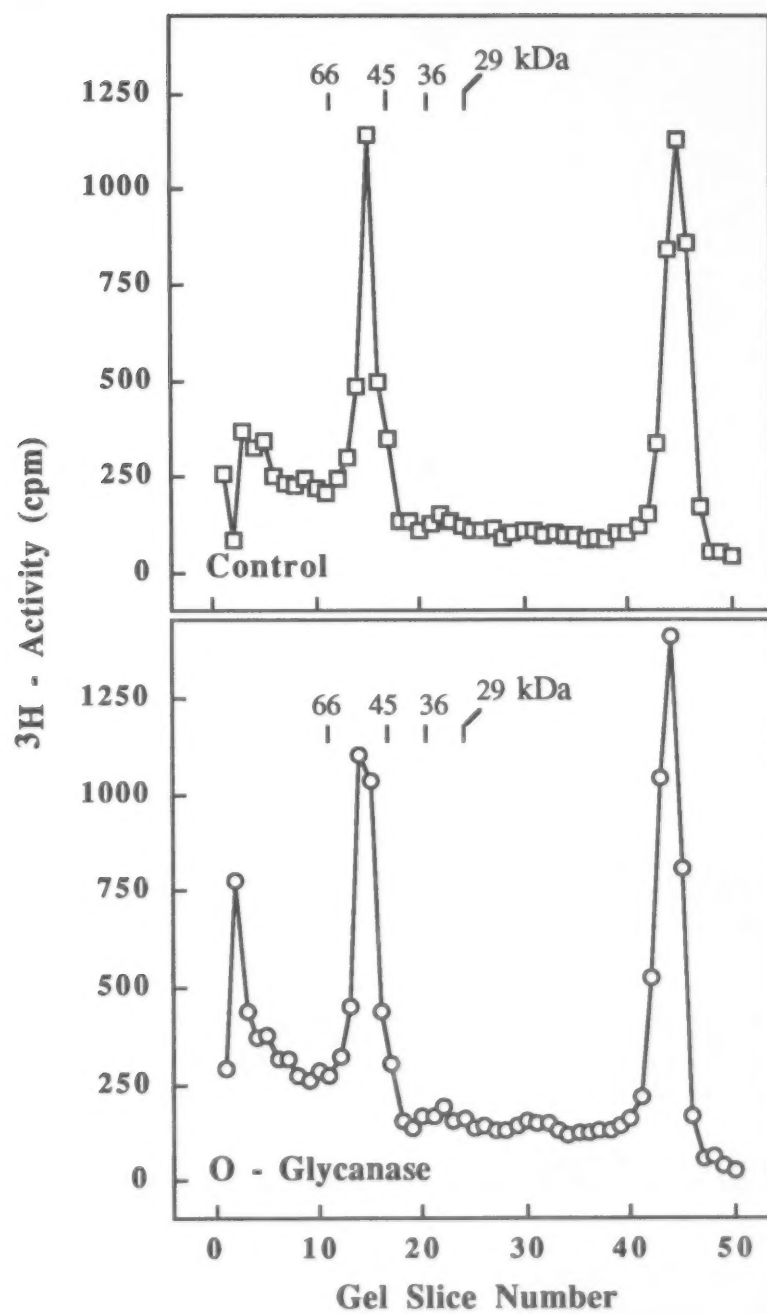


FIGURE II 20

The N-Linked Oligosaccharide of L1210/C2 Plasma Membrane [³H]NBMPR-Labeled Protein is Sensitive to Digestion by N-Glycanase, But Not by Endoglycosidase F

[³H]NBMPR-photolabeled plasma membrane fractions were prepared from L1210/C2 cells and (i) solubilized in N-glycanase digestion buffer ($\approx 250 \mu\text{g}/\text{digestion reaction}$) and incubated in the absence or presence of 1.5 U of N-glycanase (37°C, 17 hr) or (ii) resuspended in endoglycosidase F digestion buffer ($\approx 250 \mu\text{g}/\text{digestion reaction}$) and incubated in the absence or presence of 1.5 U endoglycosidase F at 37°C for 17 hr. The reaction mixtures were solubilized under reducing conditions and subjected to electrophoresis on a 12% SDS-polyacrylamide gel. Migration of photolabeled proteins was determined by slicing lanes into 2 mm slices and estimating the ³H-content in each slice by liquid scintillation counting. The positions of molecular weight standards were determined by staining with Coomassie blue and are indicated at the top of the figure.

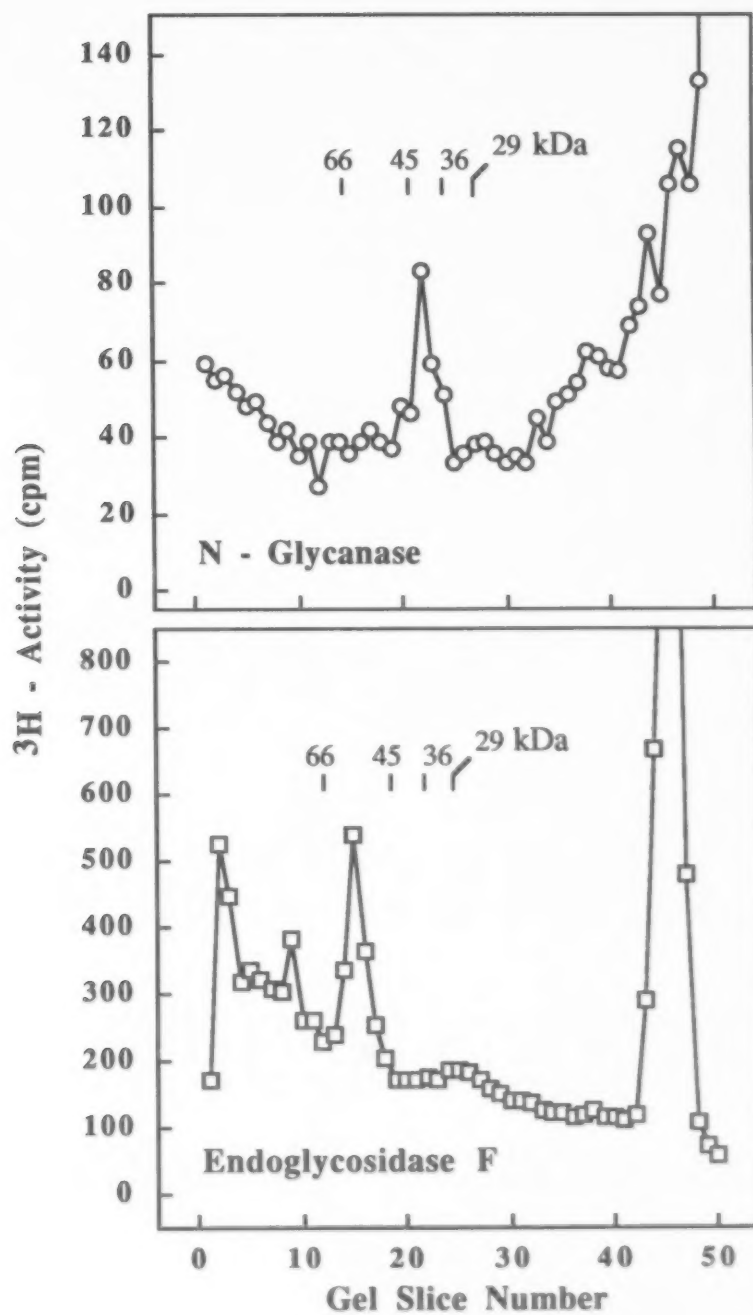
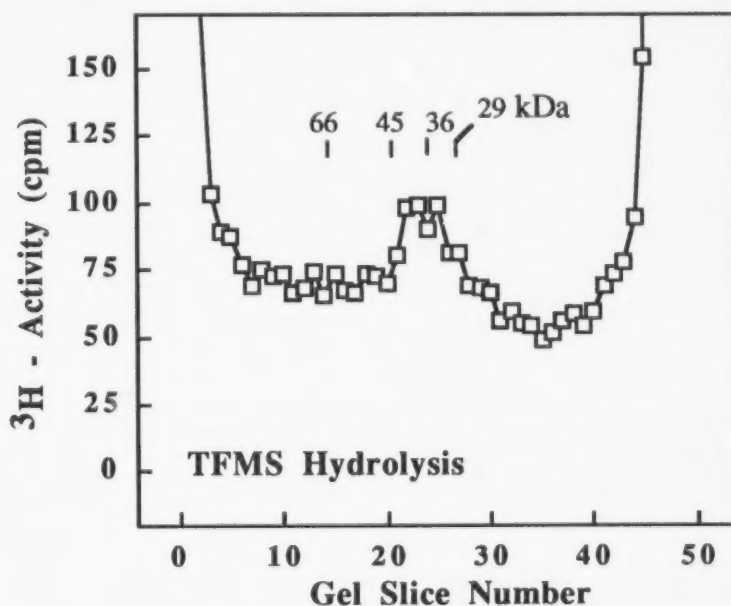


FIGURE II 21

Chemical Hydrolysis of Oligosaccharide Present on the L1210/C2 Plasma Membrane [^3H]NBMPR-Labeled Protein Generates Lower Molecular Weight Species

Portions (500 μg protein) of [^3H]NBMPR-photolabeled plasma membrane fractions prepared from L1210/C2 cells were lyophilized and subjected to chemical hydrolysis (2.5 hr, 4°C) with trifluoromethanesulfonic acid (TFMS) as described in Materials and Methods. The reaction mixtures were solubilized under reducing conditions and subjected to electrophoresis on a 12% SDS-polyacrylamide gel. Migration of photolabeled proteins was determined by slicing lanes into 2 mm slices and estimating the ^3H -content in each slice by liquid scintillation counting. The positions of molecular weight standards were determined by staining with Coomassie blue and are indicated at the top of the figure.



BIBLIOGRAPHY

- Abeijon, C. and Hirschberg, C.B. (1992) Topography of glycosylation reactions in the endoplasmic reticulum. *Trends in Biol. Sci.* **17**, 32-36.
- Alboim, S. V., Bak, A., and Sampson, S. R. (1992) Tunicamycin reduces Na^+ - K^+ pump expression in cultured skeletal muscle. *J. Cell. Physiol.* **150**, 640-646.
- Asano, T., Katagiri, H., Takata, K., Lin, J. L., Ishihara, H., Tsukuda, K., Kikuchi, M., Hirano, H., Yazaki, Y., (1991) The role of N-glycosylation of GLUT1 for glucose transport activity. *J. Biol. Chem.* **266**, 24632-24636.
- Belsham, G.J., Denton, R.M., and Tanner, M.J. (1980) Use of a novel rapid preparation of fat-cell plasma membranes employing Percoll to investigate the effects of insulin and adrenaline on membrane protein phosphorylation within intact fat-cells. *Biochem. J.* **192**, 457-467.
- Belt, J. A. (1983) Two types of transport activity in L1210 and other cultured neoplastic cells. *Mol. Pharm.* **24**, 479-484.
- Boege, F., Ward, M., Jurss, R., Hekman, and Helmreich, E. J. M. (1988) Role of glycosylation for β 2-adrenoreceptor function in A431 cells. *J. Biol. Chem.* **263**, 9040-9049.
- Boleti, B. (1991) Nucleoside transport in K562 leukemia cells. Ph.D. thesis, Department of Biochemistry, University of Alberta, Edmonton, Alberta.
- Bradford, M.M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.
- Bylund, D.B. (1980) Analysis of receptor binding data. In: *Receptor Binding Techniques* Society for Neuroscience, Bethesda MD.
- Cass, C.E. and Au-Yeung, T.H. (1976). Enhancement of 9- β -D-arabinofuranosyladenine cytotoxicity to mouse leukemia L1210 in vitro by 2'-deoxycoformycin. *Cancer Res.* **36**, 1486-1491.
- Cass, C.E., Dahlig, E., Lau, E.Y., Lynch, T.P., and Paterson, A.R.P. (1979) Fluctuations in nucleoside uptake and binding of the inhibitor of nucleoside transport, nitrobenzylthioinosine, during the replicative cycle of HeLa cells. *Can. cer Res.* **39**, 1245-1252.
- Cass, C.E., Gaudette, L.A., and Paterson, A.R.P. (1974) Mediated transport of nucleosides in human erythrocytes: specific binding of the inhibitor nitrobenzylthioinosine to nucleoside transport sites in the erythrocyte membrane. *Biochim. Biophys. Acta.*, **345**, 1-10.
- Cass, C.E., Kolassa, N., Uehara, Y., Dahlig-Harley, E., Harley, E.R., and Paterson, A.R.P. (1981) Absence of binding sites for the transport inhibitor nitrobenzylthioinosine to nucleoside transport-deficient mouse lymphoma cells. *Biochim. Biophys. Acta.* **649**, 769-777.

Cass, C.E., Tan, T.H., and Selner, M. (1979) Antiproliferative effects of 9- β -D-arabinofuranosyladenine 5'-monophosphate and related compounds in combination with adenosine deaminase inhibitors against mouse leukemia L1210/C2 cells in culture. *Can.cer Res.* **39**, 1563-1569.

Chello, P. L., Sirotak, F. M., Dorick, D. M., Yang, C. H., and Montgomery, J. A. (1983) Initial rates and evidence for duality of mediated transport of adenosine, related purine nucleosides, and nucleoside analogues in L1210 cells. *Can.cer Res.* **43**, 97-103.

Crawford, C.R., Ng, C.Y.C., and Belt J.A. (1990) Isolation and characterization of an L1210 cell line retaining the sodium-dependent carrier *cif* as its sole nucleoside transport activity. *J. Biol. Chem.* **265**, 13730-13734.

Dagnino, L (1988) Sodium-driven nucleoside transport in mouse leukemia L1210 cells. Ph.D. thesis, Department of Pharmacology, University of Alberta, Edmonton, Alberta.

Dagnino, L. and Paterson, A. R. P. (1990) Sodium-dependent and equilibrative nucleoside transport systems in L1210 mouse leukemia cells: effect of inhibitors of equilibrative systems on the content and retention of nucleosides. *Cancer Res.* **50**, 6549-6553.

Dunham, L.J. and Stewart, H.L. (1953). A survey of transplantable and transmissible animals tumors. *J. Natl. Can. Instit.* **13**, 1299-1377.

Elbein, A. D. (1987) Glycosylation inhibitors for N-linked glycoproteins. *Meth. Enzymol.* **138**, 661-709.

Gati, W.P., Belt, J.A., Jakobs, E.S., Young, J.D., Jarvis, S.M., and Paterson, A.R.P. (1986) Photoaffinity labelling of a nitrobenzylthioinosine-binding polypeptide from cultured Novikoff hepatoma cells. *Biochem. J.* **236**, 665-670.

Good, A. H., Craik, J. D., Jarvis, S. M., Kwong, F. Y. P., Young, J. D., Paterson, A. R. P., and Cass, C. E. (1987) Characterization of monoclonal antibodies that recognize band 4.5 polypeptides associated with nucleoside transport in pig erythrocytes. *Biochem. J.* **244**, 749-755.

Hakimuddin, T. S. and Bahl, O. P. (1987) Chemical deglycosylation of glycoproteins. *Meth. Enzymol.* **138**, 341-350.

Harley, E.R., Paterson, A.R.P., and Cass, C.E. (1982) Initial rate kinetics of the transport of adenosine and 4-amino-7-(β -D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine (tubercidin) in cultured cells. *Cancer Res.* **42**, 1289-1295.

Hart, G. W. (1992) Glycosylation. *Curr. Opin. Cell Biol.* **4**, 1017-1023.

Hunston, D.L. (1975) Two techniques for evaluating small molecule-macromolecule binding in complex system. *Anal. Biochem.* **63**, 99-109.

Hunt, R. C., Reigler, R., and Davis, A. A. (1989) Changes in glycosylation alter the affinity of the human transferrin receptor for its ligand. *J. Biol. Chem.* **264**, 9643-9648.

Jarvis, S. M. and Martin, B. W. (1985) Effects of temperature on the transport of nucleosides in guinea pig erythrocytes. *Can. J. Physiol. Pharmacol.* **64**, 193-198.

- Jarvis, S. M. and Young, J. D. (1987) Photoaffinity labelling of nucleoside transporter polypeptides. *Pharmac. Ther.* **32**, 339-359.
- Jones, E. W. and Fink G. R. (1982) Regulation of amino acid and nucleotide biosynthesis in yeast. In: *The molecular biology of the yeast Saccharomyces*, metabolism and gene expression. pp. 181-299. Cold Spring Harbor Laboratory
- Kalyan, N.K. and Bahl, O.P. (1983) Role of carbohydrate in human chorionic gonadotropin. *J. Biol. Chem.* **258**, 67-74.
- Keegan, A. D. and Conrad, D. H. (1987) The murine lymphocyte receptor for IgE V. Biosynthesis, transport, and maturation of the B cell Fc ϵ receptor. *J. Immunol.* **139**, 1199-1205.
- Khyim, J.X. (1975) An analytical system for rapid separation of tissue nucleotides at low pressures on conventional anion exchanges. *Clin. Chem.* **21**, 1245-1252.
- Kornberg, R. and Kornberg, S. (1985) Assembly of asparagine-linked oligosaccharides. *Ann. Rev. Biochem.* **54**, 631-664.
- Kwong, F. Y. P., Baldwin, S. A., Scudder, P. R., Jarvis, S. M., Choy, M. Y. M., and Young, J. D. (1986) Erythrocyte nucleoside and sugar transport. *Biochem. J.* **240**, 349-356.
- Kwong, F. Y. P., Fincham, H. E., Davies, A., Beaumont, N., Henderson, P. J. F., Young, J. D., and Baldwin, S. A. (1992) Mammalian nitrobenzylthioinosine-sensitive nucleoside transport proteins. *J. Biol. Chem.* **267**, 21954-21960.
- Kwong, F. Y. P., Wu, J. S. R., Shi, M. M., Fincham, H. E., Davies, A., Henderson, P. J. F., Baldwin, S. A., and Young, J. D. (1993) Enzymatic cleavage as a probe of the molecular structures of mammalian equilibrative nucleoside transporters. *J. Biol. Chem.* **268**, 22127-22134.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of bacteriophage T4. *Nature* **227**, 680-685.
- Lauzon, G.J. and Paterson, A.R.P. (1977) Binding of the nucleoside transport inhibitor nitrobenzylthioinosine to HeLa cells. *Mol. Pharmacol.* **13**, 883-891.
- Leconte, I., Auzan, C., Debant, A., Rossi, B., and Clauser, E. (1992) N-linked oligosaccharide chains of the insulin receptor β subunit are essential for transmembrane signaling. *J. Biol. Chem.* **267**, 17415-17423.
- Maher, F. and Harrison, L. C. (1991) Stimulation of glucose transporter (GLUT1) mRNA and protein expression by inhibitors of glycosylation. *Biochim. Biophys. Acta.* **1089**, 27-32.
- Marz, R., Wohlhueter, R.M., and Plagemann, P.G.W. (1979) Purine and pyrimidine transport and phosphoribosylation and their interaction in overall uptake by cultured mammalian cells. *J. Biol. Chem.* **254**, 2329-2338.

Morin, M.J., Porter, C.W., McKernan, P., and Bernacki, R.J. (1983) The biochemical and ultrastructural effects of tunicamycin and D-glucosamine in L1210 cells. *J. Cell. Physiol.* **114**, 162-172.

Nageswara, R.T. and Bahl, O.P. (1987) Enzymatic deglycosylation of glycoproteins. *Meth. Enzymol.* **138**, 350-359.

Olden, K., Pratt, R. M., Jaworski, C., and Yamada, K. M. (1979) Evidence for role of glycoprotein carbohydrates in membrane transport: specific inhibition by tunicamycin. *Proc. Natl. Acad. Sci. USA* **76**, 791-795.

Ott, R. J., Hui, A. C., and Giacomini, K. M. (1992) Inhibition of N-linked glycosylation affects organic cation transport across the brush border membrane of opossum kidney (OK) cells. *J. Biol. Chem.* **267**, 133-139.

Pande, S.V. (1976) Liquid scintillation counting of aqueous samples using Triton-containing scintillants. *Anal. Biochem.* **74**, 25-34.

Paterson, A.R.P., Harley, E.R., and Cass, C.E. (1984) Inward fluxes of adenosine in erythrocytes and cultured cells measured by a quenched-flow method. *Biochem. J.* **224**, 10001-10008.

Plagemann, P.G.W. and Wohlheuter, R. M. (1984) Effect of temperature on kinetics and differential mobility of empty and loaded nucleoside transporter of human erythrocytes. *J. Biol. Chem.* **259**, 9024-9027.

Plagemann, P.G.W. and Kraupp, M. (1986) Inhibition of nucleoside and nucleobase transport and nitrobenzylthioinosine binding by dilazep and hexobendine. *Biochem. Pharmacol.* **35**, 2559-2567.

Plagemann, P. G. W., Wohlheuter, R. M., and Woffendin, C. (1988) Nucleoside and nucleobase transport in animal cells. *Biochim. Biophys. Acta.* **947**, 405-433.

Prives, J. and Bar-Sagi, D. (1983) Effect of tunicamycin, an inhibitor of protein glycosylation, on the biological properties of acetylcholine receptor in cultured muscle cells. *J. Biol. Chem.* **258**, 1775-1780.

Pulido, R. and Sanchez-Madrid, F. (1992) Glycosylation of CD45: carbohydrate processing through the golgi apparatus is required for cell surface expression and protein stability. *Eur. J. Immunol.* **22**, 463-468.

Rademacher, T.W., Parekh, R.B., and Dwek, R.A. (1988) Glycobiology. *Ann. Rev. Biochem.* **57**, 785-838.

Roobol, C., De Dobbeleer, G.B.E., and Bernheim, J.L. (1984) 5-Fluorouracil and 5-fluoro-2'-deoxyuridine follow different pathways in the induction of cell lethality in L1210 leukemia. *Br. J. Cancer* **49**, 739-744.

Rosenthal, H.E. (1967) Graphic method for the determination and presentation of binding parameters in a complex system. *Anal. Biochem.* **20**, 525-532.

Ross, E.M., Maguire, M.E., Sturgill, T.W., Biltonen, R.L., and Gilman, A.G. (1977) Relationship between the β -adrenergic receptor and adenylate cyclase. *J. Biol. Chem.* **252**, 5761-5775.

Stein, W.D. (1986) Transport and diffusion across cell membranes. Academic Press Inc., Orlando.

Vijayalakshmi, D., Dagnino, L., Belt, J.A., Gati, W.P., Cass, C.E., and Paterson, A.R.P. (1992) L1210/B23.1 cells express equilibrative, inhibitor-sensitive nucleoside transport activity and lack two parental nucleoside transport activities. *J. Biol. Chem.* **267**, 16951-16956.

Wohlheuter, R.M. and Plagemann, P.G.W. (1980) The roles of transport and phosphorylation in nutrient uptake in cultured animal cells. *Int. Rev. Cytol.* **64**, 174-239.

Young, D. J., Jarvis, S.M., Belt, J.A., Gati, W.P., and Paterson, A.R.P. (1984) Identification of the nucleoside transporter in cultured mouse lymphoma cells. *J. Biol. Chem.* **259**, 8363-8365.

Young, D. J., Jarvis, S.M., Robins, M.J., and Paterson, A.R.P. (1983) Photaffinity labeling of the human erythrocyte nucleoside transporter by N⁶-(p-azido-benzyl)adenosine and nitrobenzylthioinosine. *J. Biol. Chem.* **258**, 2202-2208.

Yusufi, A. N. K., Szczepanska, M., and Dousa, T. P. (1988) Role of N-linked oligosaccharides in the transport activity of the Na⁺/H⁺ antiporter in rat renal brush-border membrane. *J. Biol. Chem.* **263**, 13683-13691.

Chapter III

IDENTIFICATION OF A MURINE PROTEIN THAT FUNCTIONALLY COMPLEMENTS A NUCLEOSIDE TRANSPORTER CLONING METHOD IN YEAST¹

¹ A version of this chapter has been submitted for publication: Hogue, D. L. , Ellison, M. J., Young, J. D. and Cass, C. E.

INTRODUCTION

Proteins that mediate equilibrative NT processes in the plasma membranes of mammalian cells have proven difficult to isolate and characterize. The *es* NT protein of human erythrocytes has been purified to homogeneity and used to generate antibodies (Kwong *et al.*, 1988), however the actual structure of this protein and the identity of its cDNA sequence remains unknown. No information whatsoever exists concerning either the sequence of the genes or the structure of the proteins responsible for *ei* NT processes. Since traditional biochemical approaches used for the identification of sequences of equilibrative NT proteins and their genes have so far proved unsuccessful, an alternate method for the identification of these NT proteins based on their functional expression was undertaken.

The identification and isolation of a cDNA-encoded mammalian plasma membrane protein by functional expression in mammalian cells was initially described by Seed and Aruffo (1987). Subsequently, many different receptor cDNAs have been isolated by methods that are based on the capacity of host cells expressing the recombinant receptor to bind radioactively labeled ligand. An essential requirement for such a cloning approach is the complete absence of the receptor in question, or of a related receptor capable of binding the radioactively-labeled ligand, in the host cells. Similarly, a host cell type to be used for the identification of recombinant equilibrative NT proteins by a functional expression cloning approach should lack the capacity for NT. However, most cell types examined to date express one or more types of NT process and are not suitable for use. The murine lymphoma AE₁ mutant cell line lacks a NT process (Cohen *et al.*, 1979) and thus constitutes a potential host cell type. Unfortunately, AE₁ cells are extremely fragile and did not survive the DEAE-dextran- or liposome-mediated transfection methods normally employed in functional expression cloning².

The yeast *Saccharomyces cerevisiae* lacks a mediated process for thymidine flux (Grenson, 1969) and thus represents a host cell type for use in expression cloning. This yeast also shares many cellular processes and pathways with higher eucaryotic cell types and is able to functionally express mammalian plasma membrane proteins such as the thyroid hormone (Lu *et al.*, 1990), acetylcholine (Fujita *et al.*, 1986), vitamin D (Sone *et al.*, 1990) and progesterone receptors (Mak *et al.*, 1989), and also the *mdr1* and *mdr3* isoforms of P-glycoprotein (Kuchler and Thorner, 1992; Raymond *et al.*, 1992). Recently, functional expression cloning approaches using *S. cerevisiae* as the host cell allowed for the identification of *Arabidopsis thaliana* cDNA that encode the sucrose (Reismeyer *et al.*, 1992), neutral amino acid (Hsu *et al.*, 1993) and general amino acid transporters (Frommer *et al.*, 1993), as well as a K⁺ channel (Sentenac *et al.*, 1992). In light of this evidence, *S. cerevisiae* appeared to be suitable as a host cell for the identification of a mammalian NT protein by expression cloning.

This chapter describes the development of a functional cloning methodology that utilizes *S. cerevisiae* to identify and isolate cDNA-encoded proteins which mediate thymidine influx across the plasma membrane. *S. cerevisiae* lacks a thymidine salvage pathway which includes a (i) thymidine influx process and (ii) thymidine kinase (TK). Therefore, in the presence of exogenous thymidine yeast fail to survive when *de novo* dTMP synthesis is inhibited. A strain of TK-expressing yeast was generated for use in an expression cloning method that took advantage of the absence of a thymidine influx process. It was hypothesized that TK-expressing yeast expressing a recombinant NT protein should survive under conditions wherein *de novo* dTMP synthesis was inhibited and exogenous thymidine was available, thereby allowing for isolation of cDNA that encode NT proteins. In an attempt to identify such cDNAs of equilibrative NT proteins, a

² Unpublished observations of D. Hogue and C. Cass.

L1210/C2 expression library was subjected to functional screening in *S. cerevisiae* by this method.

METHODS AND MATERIALS

Materials

Cell culture media (Fisher's, RPMI 1640, DMEM, MEM), calf serum, horse serum, fetal bovine serum, and gentamycin were obtained from Gibco Laboratories; NuSerum was from Collaborative Biomedical.; [γ - ^{32} P]ATP, [α - ^{32} P]dCTP, [α - ^{35} S]dATP and Trans ^{35} S-Label were from DuPont Canada Inc.; D-[5,6- ^{3} H]uridine, D-[methyl- ^{3} H]thymidine, D-[3,8- ^{3} H]adenosine and EcoLite scintillant were from ICN Biomedicals Inc.; light paraffin oil (Saybolt Viscosity 125-135) was from Fisher Scientific; silicon 550 oil was from Dow Corning Corp.; dilazep hydrochloride was a gift from F. Hoffman-LaRoche; restriction enzymes and DNA modification enzymes were from Gibco/BRL or New England Biolabs; Bacto-Yeast Extract, Bacto-Casamino acids, Bacto-Peptone, Difco Bacto-Agar, and Bacto-Yeast Nitrogen Base were from Difco Inc.; sulfanilamide and methotrexate were from Sigma Chemicals; the λ FIX II mouse spleen genomic DNA library and pBlueScript vectors were from Stratagene Inc.; Low Range SDS-PAGE prestained protein molecular weight standards were from BioRad; the neonatal mouse (strain C57BL/6J) brain λ GT11 library was from John Gardner (Fox Chase Cancer Center, Philadelphia, PA); Hybond-N membrane was from Amersham Life Sciences; the rabbit reticulocyte lysate system was from Promega; and other materials were obtained from standard commercial sources.

Mammalian Cell Lines

The L1210/C2 cell line (Cass and Au-Yeung, 1976) originated from a DBA/2 mouse (Dunham and Stewart, 1953) and was used as a source of polyA⁺ RNA for the construction of the cDNA expression library. The murine leukemia L1210/B23 (Vijayalakshmi *et al.*, 1992) and L1210/MA27 (Crawford *et al.*, 1990a) cell lines are nucleoside transport mutants of L1210/B2 cells (Crawford *et al.*, 1990b). The S49 mouse T-cell lymphoma cell line and its nucleoside transport-deficient AE₁ clone (Cohen *et al.*, 1979) were originally obtained from S. Martin (UCSF). The Walker 256 (W256) rat carcinosarcoma cells were provided by A.R.P. Paterson (University of Alberta). The mouse and rat cell lines were maintained as suspension cultures in RPMI 1640 medium supplemented with NaHCO₃ (2 g/l) and 10% horse serum. Human HL60 promyelocytic leukemia cells, originally obtained from R. Gallo (National Institute of Health), and HeLa S3 human epitheloid carcinoma cells (American Type Culture Collection, ATCC) were grown in RPMI 1640 containing 15% fetal bovine serum/NaHCO₃ (2 g/l) and MEM containing 10% calf serum/NaHCO₃ (2 g/l), respectively. Stock cultures were maintained in media without antibiotics at 37°C in 95% air and 5% CO₂ with 95% humidity. Cell concentrations of cultures were determined using a Coulter Counter Model ZF electronic particle counter (Coulter Electronics Inc.) and stock suspension cultures were diluted to 0.1×10^5 - 0.2×10^5 cells/ml every 2-3 days. Adherent cultures were subcultured to 0.1×10^5 - 0.2×10^5 cells/ml every 4-6 days. Cell cultures were maintained for a maximum of 30-40 generations, then discarded. Large volumes of cell cultures were generated in 1330 cm² glass roller bottles (50 rpm, Bellco) in growth medium that usually contained gentamycin (50 μ g/ml). Experiments in this study utilized

cell cultures that exhibited exponential cell proliferation (density $\leq 7 \times 10^5$ cells/ml or $\leq 70\%$ estimated confluency).

***Escherichia coli* Strains**

Plasmid propagation was carried out in DH5 α (*supE44*, *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*) or XL1-Blue (*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac[F', proAB, LacI Δ Z Δ M15, Tn10(*ter'*)]*). LE392 (*e14* [*mcrA*], *hsdR514*, *supE44*, *supF58*, *lacY1*, *galT22*, *metB1*, *trpR55*) was used for propagation of bacteriophage libraries. All strains of *E. coli* were grown in Luria Broth (LB; 10 g/l Bacto-Tryptone, 5 g/l Yeast Extract, 5 g/l NaCl) or on LB plates (LB containing 2% Bacto-agar (w/v)) at 37°C, unless otherwise stated. Plasmid maintenance was achieved by inclusion of ampicillin (50 mg/l) in broth or plate media.

***Saccharomyces cerevisiae* Strains**

The yeast strain KY114 (α , *gal*⁺, *ura3-52*, *trp1*, *lys2*, *ade2*, *his d200*) was obtained from M. Ellison (University of Alberta). Culture media used in this study were prepared according to Sherman *et al.* (1986) and included (i) Yeast-Peptone-Dextrose (YPD; 2% (w/v) glucose, 1% (w/v) Bacto-Yeast Extract, 1% (w/v) Bacto-Peptone), (ii) Yeast-Peptone-Galactose (YPG; 2% (w/v) galactose, 1% (w/v) Bacto-Yeast Extract, 1% (w/v) Bacto-Peptone), and (iii) Complete Minimal Medium (CMM; 0.7% (w/v) Bacto-Yeast Nitrogen Base without amino acids, 20 mg/l of following amino acids; histidine, adenine, uracil, lysine, tryptophan) that contained either 2% (w/v) glucose or galactose. Plating medium was prepared by the inclusion of 2% (w/v) Difco Bacto-Agar in medium. Plasmids were maintained in yeast strains by growth on CMM that lacked uracil and/or tryptophan.

Permeant Uptake Studies in *S. cerevisiae*

Sterile stock solutions of non-radioactive permeants and NT inhibitors were prepared by dissolving the compound in sterile dH₂O and filtering through a sterile 0.2 μ m Millex-GV filter (Millipore). All solutions were stored at -20°C. The concentrations of nucleoside stock solutions were determined by UV absorbance using the molar extinction coefficient ($\bar{\epsilon}$) of the compounds at a wavelength of maximum absorption ($\lambda_{\max}$), as listed below. Radiolabeled nucleosides were purified with a Varian 5000 high-pressure liquid chromatography instrument using water-methanol gradients on a C18 reverse phase column, as described in detail elsewhere (Boleti, 1991). Purified nucleosides were stored as water-methanol solutions at 4°C and were used within 2 months of purification date.

Compound	$\lambda_{\max}$ (nm)	$\bar{\epsilon}$ (mM ⁻¹ cm ⁻¹)
Ado	259	15.4
Urd	262	10.1
dThd	267	9.73
NBMPR	290	25.1
Dilazep	266	18.42

Actively proliferating cultures of KY114 (OD₆₀₀ <1.5) grown in YPD were utilized for permeant uptake studies (1.0 OD₆₀₀ $\approx 2 \times 10^7$ cells/ml). Cells were pelleted by centrifugation (2500 x g, 5 min), suspended at an OD₆₀₀ = 0.80 in 2% (w/v) glucose/CMM that contained 100 μ M [³H]nucleoside, and incubated (shaking) at 30°C. Triplicate portions (200 μ l) of yeast cultures were removed at various time points after

pellet suspension and layered over a 250 μ l volume of paraffin/silicon oil mixture in microcentrifuge tubes. The oil mixture was prepared by mixing approximately 75 ml paraffin oil (Saybolt Viscosity 125-135) with 425 ml silicon 550 oil. The final specific gravity (Specific Gravity/Baume Hydrometer) was adjusted to 1.032 g/ml using either type of oil. Uptake intervals were terminated by pelleting the cells through the oil layer by centrifugation (9000 x g, 2 min). Aqueous layers in the assay tubes were removed and the tube walls were washed twice with water. The oil layers were then removed and the cell pellets were dissolved in 250 μ l of 5% Triton X-100 by extensive vortexing. The tubes were placed in scintillation vials, 5 ml EcoLite scintillant was added and radioactive content was measured.

Measurement of Thymidine Kinase Activity in *S. cerevisiae*

The presence of thymidine kinase (TK) in yeast was assessed using an adaptation of the method of McNeil and Friesen (1981) in which dTMP produced from dThd is selectively adsorbed to DEAE-cellulose disks. Yeast cultures (50 ml) were grown to an OD₆₀₀ of 0.8 and harvested by centrifugation (2500 x g, 5 min). The cells were resuspended in 500 μ l of "TK lysis buffer" (0.2 mM ATP, 1.4 mM β -mercaptoethanol, 10 mM Tris-HCl pH 8.0) and centrifuged again. The cells were resuspended in 500 μ l of TK lysis buffer and an equal volume of nitric acid-washed glass beads (425-600 μ m diameter) was added. The cell-bead mixtures were vortexed vigorously for 5 min and subjected to centrifugation (12000 x g, 20 min, 4°C). The supernatant (cell extract) was recovered for assay of TK activity. The assay was initiated by combining 100 μ l of cell extract, 100 μ l of dH₂O and 200 μ l of "TK assay buffer" (10 mM NaF, 10 mM CaCl₂, 20 mM ATP, 20 mM β -mercaptoethanol, 0.01 μ Ci/ μ l [³H]dThd, 100 mM Tris-HCl pH 8.0) in a microcentrifuge tube and incubating at 37°C. In some tubes, the 100- μ l portion of dH₂O was substituted with 100 μ l of 1 mM competing substrate (dUrd, dCyt, dTTP) in dH₂O. Portions (100 μ l) of this mixture were removed after varying periods of incubation and transferred to microcentrifuge tubes, which were immediately immersed in boiling H₂O for 2 min. The tube contents were centrifuged (12000 x g, 2 min, 4°C) and 50- μ l portions of supernatant were applied to DE81 DEAE-cellulose disks (Whatman), which were subsequently allowed to dry at RT for 4 hr. The disks were successively washed twice in 5-ml volumes of 4.0 mM ammonium formate and 95% ethanol and then placed into scintillation vials. EcoLite scintillation fluid (5 ml) was added to each vial and the radioactivity was measured by liquid scintillation counting.

Isolation of RNA

Glassware used for RNA isolation was baked at 425°F for 4 hr and all solutions, with the exception of Tris-containing solutions, were treated with diethylpyrocarbonate (DEPC) according to Ausubel *et al.* (1989). Total cellular RNA was isolated from cultured mammalian cells by the method of Chirgwin *et al.* (1979). Cells were pelleted by centrifugation (450 x g, 5 min) and then dissolved (10⁹ cells/15 ml) in guanidinium thiocyanate solution (4 M guanidinium thiocyanate, 0.5% sodium lauryl sarcosine, 0.1 M β -mercaptoethanol, 25 mM sodium citrate, pH 7.0). CsCl was added (1 g per 2.5 ml of cell-guanidinium solution) and the solution was layered onto a cushion of 5.7 M CsCl in 0.1 M EDTA (pH 7.0) and subjected to centrifugation (20000 x g, 21 hr, 20°C). The resulting RNA pellet was resuspended in Tris-EDTA buffer (TE buffer, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and extracted with an equal volume of phenol:CHCl₃:isoamyl alcohol (25:24:1, v/v). RNA was precipitated by the addition of 0.1 volume of 3.0 M sodium acetate (pH 6.0) and 2.5 volumes of 100% ethanol. A different method was used to isolate smaller quantities of RNA (Ausubel *et al.*, 1989). Cell pellets were dissolved in guanidinium thiocyanate solution (10⁷ cells/ml) and the suspension was mixed with 0.1 volume of 2.0 M sodium acetate (pH 4), after which was added an equal volume of

dH₂O-saturated phenol and 0.05 volume of 49:1 (v/v) CHCl₃/isoamyl alcohol. The resulting solution was centrifuged (9000 x g, 20 min, RT), the top aqueous layer was recovered, and RNA was precipitated by the addition of an equal volume of isopropanol followed by centrifugation (9000 x g, 20 min, RT). The RNA pellet was dissolved in 0.3 ml of guanidinium thiocyanate solution and reprecipitated with isopropanol. RNA was stored at -70°C as an isopropanol precipitate.

Total RNA was isolated from *S. cerevisiae* using a glass bead disruption procedure (Ausubel *et al.*, 1989) carried out at 4°C. Cells were pelleted by centrifugation (2500 x g, 5 min), washed once in ice-cold dH₂O, and pelleted again by centrifugation. The cells were suspended in RNA buffer (0.5 M NaCl, 10 mM EDTA, 200 mM Tris-HCl pH 7.5) at a concentration of 2 x 10⁸ cells/ml and subjected to centrifugation (9000 x g, 5 sec). The cells were resuspended in RNA buffer at 0.3 volume of the previous suspension and mixed with 0.5 volumes of nitric acid-washed glass beads (425-600 µm diameter) and phenol/CHCl₃/isoamyl alcohol (25:24:1, v/v). The cell suspension was vigorously vortexed for 2 min and centrifuged (9000 x g, 1 min). The supernatant was extracted with an equal volume of phenol/CHCl₃/isoamyl alcohol (25:24:1, v/v) and RNA was precipitated by the addition of 2.5 volumes of 100% ethanol.

Isolation of PolyA⁺ RNA

Large scale isolation of polyA⁺ RNA from total RNA was performed by chromatography on oligo(dT) cellulose (Ausubel *et al.*, 1989). Oligo(dT) cellulose (Pharmacia, Type 7) was washed with 0.1 M NaOH and packed into a silanized column that contained a silanized glass wool plug using loading buffer (0.5 M LiCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1% SDS). Total RNA was dissolved in DEPC-treated dH₂O (70°C, 10 min) and 10 M LiCl was added to a final concentration of 0.5 M LiCl. The RNA solution was passed through the column and eluate was collected and reapplied twice to the column with heating (70°C, 10 min) before each application. The column was washed with three column volumes of wash buffer (0.15 M LiCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1% SDS) and the bound polyA⁺ RNA was eluted by the addition of elution buffer (2 mM EDTA, 0.1% SDS; 60°C). Eluted polyA⁺ RNA was precipitated by the addition of 0.1 volumes of 3.0 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol.

Isolation of DNA From Bacteria

Plasmid DNA for use in restriction enzyme analysis and subcloning was prepared using a modification of the alkaline lysis mini-prep procedure (Birnboim and Doly, 1979). A 3-ml portion of bacterial culture was centrifuged (9000 x g, 5 sec) and the cells were resuspended in 0.1 ml Glucose-Tris-EDTA buffer (GTE; 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0). The cell suspension was mixed gently, by inversion, with 0.2 ml lysis solution (0.2 M NaOH, 1% SDS) and incubated at 4°C for 5 min. After an addition of 0.15 ml acetate solution (1:1.72 (v/v) of 5 M CH₃COOK/5 M CH₃COOH), the tube contents were mixed by gentle inversion and centrifuged (9000 x g, 5 min, 4°C). The supernatant (~0.35 ml) was recovered and combined with 0.9 ml of ice-cold 95% ethanol prior to centrifugation (9000 x g, 5 min, 4°C). The pellet was dissolved in 0.1 ml of TE buffer and the resulting solution was mixed with 0.1 ml of 10 M LiCl and 0.05 ml of CHCl₃ and incubated at -20°C for 20 min. The solution was centrifuged (9000 x g, 10 min, 4°C) and plasmid DNA was precipitated from the aqueous layer by the addition of 3 volumes of 95% ethanol. The DNA was dissolved in TE buffer and stored at -20°C. Isolation of plasmid DNA on a large scale, for use in cDNA library transformation into yeast, was performed by scaling up this procedure accordingly.

Plasmid DNA for use as template in DNA sequencing and *in vitro* transcription reactions was prepared using a Qiagen-tip 20 Plasmid Preparation kit (Qiagen Corp.).

Bacteria from a 50-100 ml culture were pelleted by centrifugation and used to isolate plasmid DNA according to the manufacturer's instructions.

Single-stranded DNA was generated for use in DNA sequencing was prepared using a method similar to that described by Ausubel *et al.* (1989) in which the DNA insert to be sequenced was cloned into a vector that contained a single-stranded M13 origin of replication. The resulting plasmid was transformed into the XL1-Blue or UT580 strain and a single transformant colony was grown in a 5 ml volume of LB medium (containing 50 µg/ml ampicillin) to an OD₅₅₀ of 0.6. The culture was inoculated with a 15-µl portion of M13KO7 bacteriophage solution and grown for 1 hr, then a 1-ml portion of culture was added to a 25-ml volume of fresh LB medium that contained ampicillin (100 µg/ml) and kanamycin (70 µg/ml) and grown for 12-16 hr. The culture was centrifuged (3000 x g, 10 min) and the supernatant was combined with 0.2 volumes of 20% (w/v) PEG-8000 in 2.5 M NaCl. The solution was centrifuged (9000 x g, 20 min) to pellet the single-stranded DNA. The DNA was suspended in 1 ml of buffer (0.5 mM EDTA, 10 mM NaCl, 20 mM Tris-HCl pH 8.0) and sequentially extracted with equal volumes of TE-saturated phenol (twice), chloroform-saturated phenol (once), and chloroform (once). The DNA was then precipitated by the addition of 0.05 volumes of 3.0 M sodium acetate (pH 5.2) and 2.5 volumes of 95% ethanol.

Transformation of Plasmid DNA into Bacteria

Transformation was performed by the CaCl₂ method as previously described (Ausubel *et al.*, 1989). Bacteria were grown in LB medium until an OD₅₅₀ of 0.375 and then chilled on ice. The cells were pelleted by centrifugation (1600 x g, 5 min, 4°C), resuspended in 0.025 volumes of ice-cold CaCl₂ buffer (60 mM CaCl₂, 15% glycerol, 10 mM PIPES pH 7.0), and pelleted again by centrifugation. The cells were washed a second time with CaCl₂ buffer and resuspended in a small volume (1/200 of the original culture volume) of CaCl₂ buffer. Portions of competent cell suspension were frozen in liquid N₂ and stored at -70 °C. For transformation, plasmid solution (≤20 µl) was incubated with 180 µl of competent cell suspension on ice for 30 min, placed at 37°C for 5 min, and placed again on ice for 2 min. The cell suspension was diluted with 0.8 ml of LB medium and shaken at 37°C for 45 min prior to plating onto LB plates that contained the appropriate antibiotic.

Isolation of Plasmid DNA From *S. cerevisiae*

Plasmid was isolated from yeast by the method of Baker and Schatz (1987). Portions (5 ml) of yeast culture were centrifuged (2000 x g, 5 min) and the cells were washed with 1 ml yeast lysis buffer (1 M sorbitol, 60 mM EDTA, 0.1 M NaH₂PO₄ pH 7.0) prior to resuspension in 0.2 ml lysis buffer that contained 2 mg/ml Zymolyase 5000 (Kirin Brewery) and 28 mM β-mercaptoethanol. The cell suspension was incubated at 37°C for 60 min and then cells were lysed by addition of 0.4 ml of 0.2 M NaOH/1% SDS and incubation at 4°C for 5 min. The lysed cells were mixed with a 0.5 volumes of 3.0 M CH₃COOK (pH 4.8) and incubated (4°C, 5 min) prior to centrifugation (9000 x g, 5 min). The supernatant was recovered and mixed with 2.5 volumes of 95% ethanol prior to centrifugation (9000 x g, 5 min). The plasmid-containing pellet was resuspended in TE buffer and stored at -20°C.

Transformation of Plasmids into Yeast

Plasmids were introduced into yeast by either of two methods. Transformation of yeast by electroporation was according to the method of Becker and Guarente (1989). Yeast cultures (OD₆₀₀ = 1.3) grown in YPD were harvested by centrifugation (2500 x g, 5 min, 4°C) and the cells were washed three times by suspension in ice-cold dH₂O (volume equal to that of original culture) and centrifugation. Cells were resuspended in

20 ml of ice-cold 1.0 M sorbitol and centrifuged (2000 x g, 5 min, 4°C), whereupon the supernatant was removed and the cells were resuspended in a volume of ice-cold 1.0 M sorbitol equivalent to 1/100 of the original culture volume. A mixture of 20 µl of yeast suspension and 1 µl of plasmid DNA solution (1-2 µg/µl) was incubated (4°C, 5 min) and placed between the electrodes of a micro-electroporation cuvette and then subjected to a single electrical pulse (1.5 kV, 25 µF, 200 ohms) using a BRL Gene Pulser with Pulse Controller. The cell-plasmid DNA solution was mixed with 0.5 ml of 1M sorbitol and spread on agar plates that contained the appropriate selection medium.

A second method of transformation employed the polyethylene glycol (PEG)/LiCl technique described by Elble (1992). This procedure involved harvesting 1 ml portions of actively growing yeast cultures by centrifugation (9000 x g, 5 sec) and resuspending the cells with a solution that consisted of 1 µl of plasmid DNA solution (1-2 µg/µl) and 10 µl of sheared Herring sperm DNA (10 µg/µl), then mixed with a 0.5-ml portion of PLATE solution (40% (w/v) PEG 4000, 100 mM LiCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.5) and incubated overnight at RT. The cell suspension was centrifuged (9000 x g, 5 sec) and the cells were resuspended in 50 µl of sterile dH₂O and plated onto agar plates that contained the appropriate selection medium.

Methods for Enzymatic Manipulation of DNA

Restriction enzyme digestion of plasmid DNA, DNA fragments and polymerase chain reaction (PCR) products was performed as follows. Briefly, the basic reaction consisted of a mixture that contained 0.1 volumes of the appropriate React buffer (10X concentration, Gibco/BRL), 0.1 volumes of DNA solution, 0.05 volumes of restriction enzyme stock, and 0.75 volumes of sterile dH₂O. The reactions were allowed to incubate at the appropriate temperature for 1-2 hr. Reactions containing PCR products were allowed to incubate for periods of 4-12 hr.

Analysis of DNA products of restriction enzyme digestion and PCR reactions was performed by electrophoretic separation of samples on a 1% agarose gel (1% agarose (w/v), 0.05 µg/ml ethidium bromide, 2 mM EDTA, 40 mM Tris-acetate pH 8.5) in Tris-acetate-EDTA buffer (TAE; 2 mM EDTA, 40 mM Tris-acetate pH 8.5). The DNA-containing solution was mixed with a 0.16 volume of DNA loading mix (0.25% bromophenol blue (w/v), 40% sucrose (w/v)) prior to electrophoresis. The agarose concentration of the gel was increased when DNA fragments of ≤ 200 bp were expected. DNA fragments were visualized with a UV light box.

Gel purification of DNA fragments produced by restriction enzyme digestion and PCR reactions was performed after electrophoretic separation on 1% low melting point (LMP) agarose gels in TAE buffer (Favre, 1992). DNA fragments were visualized under UV light and excised in a small quantity of LMP agarose. The agarose gel fragment was mixed with an equal volume of TE buffer and incubated at 65°C for 10 min or until the agarose gel had dissolved. The aqueous solution was twice extracted with an equal volume of Tris-buffered phenol and extracted once with 0.1 volumes of 4 M LiCl. The upper phase was then mixed with 2.5 volumes of 100% ethanol and placed at -70°C overnight. DNA fragments were pelleted by centrifugation (12000 x g, 10 min, 4°C) and washed with 70% ethanol prior to being dissolved in TE buffer and stored at -20°C.

Ligation of DNA fragments was performed by combining a 0.2 volume of ligase buffer (5X concentration, Gibco/BRL), 0.1 volumes of each DNA-containing solution, 0.1 volumes of T4 DNA ligase (Gibco/BRL), and a 0.6 volume of sterile dH₂O. The ligation mixture was incubated at 14°C (≥ 6 hr) prior to use in transformation reactions. In some cases, an in-gel ligation procedure was employed. DNA fragments excised in LMP agarose were melted at 65°C, then equal portions (7.5 µl) of DNA fragments were combined and further incubated at 65°C for 5 min. The DNA mixture was incubated at 37°C for 2 min and combined with a 15-µl portion of ice-cold ligase mixture (0.4 volumes of 5X ligase buffer, 0.12 volumes of T4 DNA ligase, and 0.48 volumes of sterile

dH₂O) and incubated at 16°C for 12 hr. The in-gel ligation mixture was diluted with 70 µl of TE buffer prior to use in transformation reactions.

Radioactive DNA probes used in hybridization analyses were produced using a random primer-labeling method similar to that described by Sambrook *et al.* (1989). Briefly, 10 µl of DNA solution (2.5 ng/µl) was boiled for 5 min and placed on ice. The chilled DNA solution was mixed with 3 µl of dNTP mix (dATP, dGTP, dTTP, 1 mM each), 2 µl of reaction mix (75 ng random hexamer oligonucleotides, 100 mM MgCl₂, 900 mM HEPES pH 6.6), 4 µl [α -³²P]dCTP, and 1 µl Klenow DNA polymerase (5 U). The reaction mixture was incubated at 37°C for 30 min and heated to 65°C (10 min) prior to the precipitation of DNA by the addition of a 0.1 volume of 3 M CH₃COONa and 2.5X volumes of 95% ethanol. The ³²P-labeled DNA pellet was suspended in 100 µl TE buffer. In some cases, a commercial random primer labeling kit was employed (Stratagene Inc.).

Sequencing of DNA

Manual sequencing of plasmid and single-stranded DNA was performed by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using a Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical). Sequencing reactions were performed exactly as suggested by the manufacturer. Portions (2.5 µl) of each sequencing reaction were loaded onto a 0.4-mm 6% acrylamide-urea gel (5.7% (w/v) acrylamide, 0.3% (w/v) N,N'-methylenebisacrylamide, 7.0 M urea, 1 mM EDTA, 100 mM Tris-borate pH 8.3) and subjected to electrophoresis in buffer (100 mM Tris, 90 mM borate, 1 mM EDTA pH 8.3). The gel was soaked in fix solution (10% CH₃OH, 10% CH₃COOH) for 15 min and dried under vacuum in a slab gel dryer. Dried gels were exposed to Kodak XAR-5 film at RT for 24 hr and sequence was manually read.

In some cases, DNA template was submitted to the Dept. of Biochemistry DNA Sequencing Lab (University of Alberta) for sequencing. This facility used an Applied Biosystems Model 373A DNA Sequencer with a Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems).

Sequencing reactions were performed on complementary strands of DNA using synthesized oligonucleotide primers oriented in opposite directions. In cases where sequences obtained from complementary DNA strands were not identical, additional sequencing reactions were performed to confirm the actual sequence. Overlapping DNA sequences were aligned using the MacVector software program.

Nucleotide and Protein Sequence Database Searching

Deduced nucleotide sequences and predicted protein sequences were compared to the nucleotide sequence databases (GenBank, EMBL, Brookhaven, Kabat, Eucaryotic Promoter) and peptide sequence databases (SWISS-PROT, PIR, Brookhaven) at the National Center for Biotechnological Information (NCBI) using the BLAST algorithm (Altschul *et al.*, 1990). The BLAST algorithm is a heuristic for finding ungapped, locally optimal, sequence alignments.

Alignments of nucleotide or peptide sequences were performed using the Sequence Analysis Software GCG Package (Genetics Computer Inc.). Nucleotide and predicted protein sequences were also compared to the sequence databases of the GCG Package using the FastA algorithm of Pearson and Lipman (1988).

Northern Blot Analysis

RNA was electrophoretically separated using a denaturing formaldehyde agarose gel (Ausubel *et al.*, 1989). The RNA-containing solution (up to 11 µl) was added to a mixture that consisted of 5 µl of concentrated MOPS buffer (10 mM EDTA, 0.1 M CH₃COONa, 0.4 M MOPS pH 7.0), 8.75 µl of 37% formaldehyde, and 25 µl formamide.

The RNA sample was then heated at 55°C (15 min) and combined with 10 µl of loading buffer (0.25% bromophenol blue (w/v), 0.25% xylene cyanol (w/v), 50% glycerol (v/v), 1 mM EDTA pH 8.0) prior to loading the sample onto the agarose gel (1.0% (w/v) agarose, 1 mM EDTA, 10 mM CH₃COONa, 40 mM MOPS pH 7.0, 1.1% formaldehyde, 0.05 µg/ml ethidium bromide). The gel was run in MOPS-containing buffer (1 mM EDTA, 10 mM CH₃COONa, 40 mM MOPS pH 7.0) with constant circulation. For reference, the gel was photographed under UV light. The gel was then washed in dH₂O (5 min), denatured in 50 mM NaOH (30 min), and neutralized in 0.5 M Tris-HCl pH 7.0 (30 min). The RNA gel was capillary blotted onto Hybond-N membrane in the presence of 20X SSC and the RNA was UV-crosslinked to the membrane (StrataLinker, Stratagene Corp.).

Membranes were incubated (shaking) in pre-hybridization solution (5X SSC, 1% (w/v) SDS, 10% (w/v) dextran sulfate, 5X Denhardt's solution (100X Denhardt's: 2% (w/v) ficoll, 2% (w/v) polyvinyl-pyrrolidone, 2% (w/v) BSA, 100 µg/ml sheared Herring sperm DNA)) at 68°C for 4 hr. ³²P-Labeled DNA probe was denatured by incubation at 100°C for 15 min and chilling on ice. The denatured probe was then added to fresh pre-hybridization solution and incubated with the membrane at 68°C for at least 18 hr. The membranes were washed at 37°C in 2X SSC/0.1% SDS and 0.1X SSC/0.1% SDS and then exposed to Kodak XAR-5 film with an intensifying screen at -70°C.

Southern Blot Analysis

DNA samples were mixed with a 0.16 volume of DNA loading mix and electrophoretically separated on 1% agarose gels (1% agarose (w/v), 2 mM EDTA, 40 mM Tris-acetate pH 8.5) in TAE buffer according to Ausubel *et al.* (1989). Gels were stained with ethidium bromide and photographed under UV light. DNA was transferred to Hybond-N membrane by capillary blotting in 20X SSC and immobilized to the membrane by UV-crosslinking. The DNA blot was subjected to hybridization with denatured ³²P-labeled DNA probe using the pre-hybridization and hybridization steps described for Northern blot analysis except that the hybridization solution contained 50% formamide. Hybridization was carried out at 50°C (12 hr) and blots were washed and processed as described above.

In cases where oligonucleotides were used as ³²P-labeled probes, an alternate hybridization strategy was used (Ausubel *et al.*, 1989). Oligonucleotide (200 ng) was 5' end-labeled with ³²P by incubation (37°C, 60 min) in kinase buffer (10 mM MgCl₂, 5 mM DTT, 50 µg/ml BSA, 50 mM Tris-HCl pH 7.5) that contained 150 µCi [γ-³²P]ATP and T4 polynucleotide kinase (20 U). The mixture was then heated to 75°C for 10 min to inactivate the T4 kinase. Membranes to be probed were incubated in pre-hybridization solution (6X SSC, 5X Denhardt's solution, 0.1% SDS, 200 µg/ml sheared Herring sperm DNA, 0.05% Na₄P₂O₇) for 2 hr at the predicted melting temperature (T_m ; $T_m = 4(G+C) + 2(A+T)$) of the oligonucleotide. The probe was added to the pre-hybridization solution, the temperature was allowed to drop slowly to 15°C below that of the predicted T_m , and then membrane was incubated at 15°C below that of the predicted T_m (4-12 hr). The membrane was washed once at RT (5 min) in 6X SSC/0.1% SDS and then subjected to 5 min washes at increasing temperatures in 6X SSC/0.1% SDS until the majority of radioactive probe had been washed off the membrane. The membrane was exposed to Kodak XAR-5 as described above.

Hybridization Screening of the Mouse Genomic Bacteriophage Library

Identification and isolation of bacteriophage containing a DNA insert of interest was performed using an established method (Sambrook *et al.*, 1989). LE392 *E. coli* cells were grown at 30°C in 50 ml broth (10 g/l Bacto-Tryptone, 5 g/l NaCl, 0.2% maltose, 10 mM MgSO₄), harvested by centrifugation (1500 x g, 10 min), resuspended in 10 mM

MgSO₄ to an OD₆₀₀ of 0.5, and stored at 4°C for up to 2 weeks. Approximately 1 x 10⁶ pfu of a λ FIX II mouse genomic DNA library (Total spleen DNA of female B6/CBA strain mouse, 6-8 weeks old; Stratagene Corp.) were mixed with 1.0 ml of LE392 cells and 50 μ l portions of this cell suspension were mixed with 6.5 ml of melted top agar (5 g/l NaCl, 5 g/l yeast extract, 10 g/l casein hydrolysate, 2 g/l MgSO₄·7H₂O, 0.7% (w/v) agarose) and plated over bottom agar (the same as top agar except with 2% agar substituted for 0.7% agarose) in 150-mm plates. The plates (20 plates at 5 x 10⁴ pfu/plate) were incubated at 37°C for 7 hr and placed at 4°C for 2 hr. Hybond-N filters were incubated (60-sec each) on the agar surface of each plate. Each filter was oriented by punching with an 18-gauge needle. The filters were then placed (plaque side-up, 2-min each) on Whatman 3MM paper that had been soaked in denaturing solution (0.15 M NaCl, 0.5 M NaOH), neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl pH 8.0), and 2X SSC. The filters were allowed to air-dry and were then subjected to UV-crosslinking. Two filters were prepared from each plate.

The filters were subjected to hybridization as described for Southern blot analysis with ³²P-labeled cDNA probe that encompassed the cDNA region of the pMTP2 plasmid. Positive plaques were identified by signals on each of the duplicate filters. Regions of agar (5 mm diameter) on plates that corresponded to signal locations were punched out and the phage were eluted by incubation (overnight, 4°C) in 100 μ l of elution buffer (5.8 g/l NaCl, 2.0 g/l MgSO₄·7H₂O, 0.1% gelatin, 50 mM Tris-HCl pH 7.5). The eluted phage were inoculated into fresh LE392 cells and additional rounds of hybridization screening were performed with a ³²P-labeled oligonucleotide probe (5' CCTGACAAAAGCTTTTCGGCCC 3') until a single isolated plaque was obtained. Phage eluted from this plaque were stored at 4°C in elution buffer.

Bacteriophage DNA was isolated from phage using a modification of the method described by Davis *et al.* (1986). A region of top agar that contained near-confluent plaques of a single positive clone was incubated (37°C, shaking) in 10 ml of broth (the same as top agar except lacking agarose) for 1-2 days. The culture was mixed with CHCl₃ (100 μ l) for 2 min to lyse bacteria and the solution was centrifuged (3000 x g, 10 min). Supernatant was recovered, mixed with 100 μ l of 1 M MgSO₄ and 10 μ l each of RNase A (10 mg/ml) and DNase I (1 mg/ml) solution and incubated at RT (15 min). The solution was then mixed with a 2-ml portion of 5 M NaCl and 2.2 g of PEG-6000 and incubated at 4°C (15 min) and then centrifuged (12000 x g, 10 min, 4°C). The phage DNA pellet was suspended in 300 μ l of buffer (10 mM MgSO₄, 50 mM Tris-HCl pH 7.4) and extracted four times with 300 μ l of CHCl₃. The DNA solution was mixed with 15 μ l of 0.5 M EDTA (pH 8.0) and 30 μ l of 5 M NaCl and then extracted sequentially with TE-buffered phenol (350 μ l) and CHCl₃ (350 μ l). Phage DNA was precipitated by the addition of 875 μ l of 95% ethanol, collected by centrifugation (12000 x g, 10 min, 4°C) and dissolved in TE buffer.

Amplification of DNA by the Polymerase Chain Reaction (PCR) Method

DNA was amplified using modifications of the polymerase chain reaction (PCR) method that employs a thermostable DNA polymerase (Saiki *et al.*, 1988). PCR reaction mixtures that employed Taq DNA polymerase consisted of the following ingredients which were combined in a 500- μ l tube at 4°C in following order: 70.5 μ l of dH₂O, 10 μ l of PCR buffer (500 mM KCl, 0.1% (w/v) gelatin, 15 mM MgCl₂, 100 mM Tris-HCl pH 8.3), 16 μ l of dNTP mix (dATP, dGTP, dTTP, dCTP; 1.25 mM each), 1 μ l of DNA template solution (1-50 pg), 1 μ l of the appropriate oligonucleotide primer solutions (25 pmol/ μ l), and finally 0.5 μ l of Taq DNA polymerase solution (5 U/ μ l). Some PCR reactions were performed using Vent DNA polymerase, a thermostable DNA polymerase with 3' → 5' proof-reading exonuclease activity (Mattila *et al.*, 1991). These reaction

mixtures, prepared under identical conditions as the Taq reaction mixtures, consisted of: 60 μ l of dH₂O, 10 μ l of PCR buffer (100 mM KCl, 20 mM MgSO₄, 0.1% (v/v) Triton X-100, 100 mM (NH₄)₂SO₄, 200 mM Tris-HCl pH 8.8), 24 μ l of dNTP mix (dATP, dGTP, dTTP, dCTP; 1.25 mM each), 1 μ l of acetylated BSA (10 mg/ml in dH₂O), 1 μ l of DNA template solution (1-50 pg), 1 μ l of the appropriate oligonucleotide primer solutions (25 pmol/ μ l), and 2 μ l of Vent DNA polymerase solution (1 U/ μ l). A portion (100 μ l) of mineral oil was layered over the aqueous reaction mixtures prior to conducting the thermal cycling steps.

The reaction mixture tubes were placed into the thermal block of thermal cycling instruments (Techne Model PHC-2 or Perkin Elmer Cetus Model 480) that had been preheated to 94°C. Samples were incubated at 94°C (2 min) prior to initiation of the thermal cycling program which usually consisted of the following cycle: denaturing at 94°C (2 min), annealing at T_m minus 5°C (1 min), and extension at 72°C (1 min per 1000 bp of expected PCR product). The annealing temperature was calculated for the oligonucleotide primer that exhibited the lower T_m and the cycle was normally repeated 20-25 times. Immediately following the thermal cycling program, the oil was removed by an extraction with an equal volume of phenol/CHCl₃ (1:1, v/v) and the PCR product was precipitated by the addition of 0.1 volume of 3 M CH₃COONa and 2.5 volumes of 95% ethanol.

Gel-purified PCR product was digested with restriction enzymes whose sites had been incorporated into the oligonucleotide primer or, in cases where no restriction sites were available, the product was blunt-ended by the following procedure. Briefly, PCR product was suspended at 37°C for 1-2 min in Klenow/kinase buffer (50 μ g/ml BSA, 1 mM DTT, 10 mM MgCl₂, 50 mM Tris-HCl pH 7.5) that contained 2.5 U of Klenow fragment, 0.5 mM ATP, and 5 U of polynucleotide kinase. A portion of dNTP mix (dATP, dGTP, dTTP, dCTP; 1.25 mM each) was added to achieve a final concentration of 0.25 mM dNTPs and the mixture was incubated at 37°C for 30 min prior and then heated (65°C, 10 min). Blunt-ended PCR product was precipitated by the addition of 0.1 volumes of 3 M CH₃COONa and 2.5 volumes of 95% ethanol.

***In Vitro* Transcription and Translation**

RNA transcript was synthesized from cDNA inserts that had been directionally cloned behind a T7 RNA promoter of the pYES2 vector. Plasmid (5 μ g) was linearized by restriction enzyme digestion at site downstream from the cDNA insert and the linearized plasmid was extracted twice with an equal volume of phenol/CHCl₃ (1:1, v/v) and precipitated by the addition of 0.1 volume of 10 M ammonium acetate and 2.5 volumes of 95% ethanol. The DNA pellet was suspended in 12 μ l of DEPC-treated dH₂O and mixed with 20 μ l of transcription buffer (250 mM NaCl, 10 mM spermidine, 40 mM MgCl₂, 200 mM Tris-HCl pH 8.0), 4 μ l of each NTP (ATP, GTP, UTP, CTP; 10 mM), 4 μ l of 750 mM DTT, 3 μ l of 40 mM m⁷G(5')ppp(5')G cap analog, 2 μ l of placental RNase inhibitor, 41 μ l of DEPC-treated dH₂O, and 2 μ l of T7 RNA polymerase solution (10 U/ μ l). The reaction was incubated at 37°C (60 min) and the plasmid DNA was then digested by an incubation (37°C, 15 min) with 1 μ l of DNase I (10 U/ μ l, RNase-free). The transcription mixture was extracted with an equal volume of phenol/CHCl₃/isoamyl alcohol (25:24:1, v/v) and precipitated using 0.1 volume of 10 M ammonium acetate and 2.5 volumes of 95% ethanol. The RNA pellet was suspended in 5 μ l of DEPC-treated dH₂O and stored at -70°C.

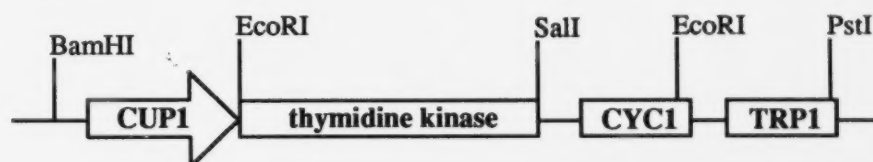
Translation of *in vitro* synthesized RNA transcript was performed using a rabbit reticulocyte lysate system. The translation reaction mixture consisted of: 17.5 μ l of rabbit reticulocyte lysate, 3.5 μ l of DEPC dH₂O, 1 μ l of placental RNase inhibitor, 0.5 μ l of 1 mM methionine-deficient amino acid mix, 2 μ l of Trans³⁵S-Label (1000 Ci/mmol), and 1 μ l of synthesized RNA transcript (1 μ g RNA). The mixture was incubated at 30°C for 60

min. The translation reaction mixture was combined with an equal volume of protein loading buffer (4% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) β -mercaptoethanol, 0.01% (w/v) bromophenol blue, 20 mM Tris-HCl pH 6.8) and subjected to single-dimension SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli (1970). Samples were electrophoretically separated on a slab gel (BioRad Protean II) using a 4% acrylamide stacking gel and separating gel of 12% acrylamide concentration. The gel was sequentially incubated (60 min) in solutions of 10% acetic acid/30% methanol/ 1% glycerol (v/v/v), EN³HANCE autoradiography enhancer (NEN Research) and cold dH₂O (15 min). The gel was then dried under vacuum and exposed to Kodak XAR-5 film at -70°C.

Construction of Expression Vectors

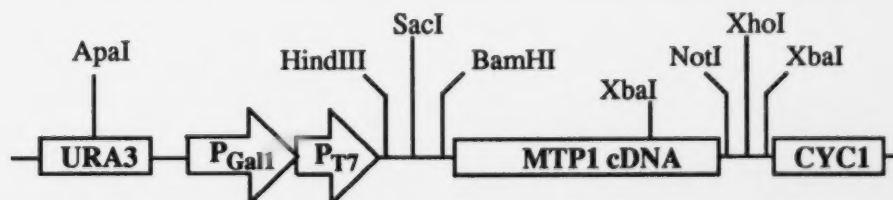
The parental vectors employed and the method of construction of expression vectors used in this study are listed below. A partial linear map indicating relevant regions of the vector and landmark restriction enzyme sites accompanies the description of each expression vector (maps are not to scale). Abbreviations are: *CUP1*, yeast copper metallothionein promoter; *CYC1*, *CYC1* transcriptional terminator; *TRP1*, 5' P-ribosyl-anthranilate isomerase gene; *URA3*, orotidine-5'-P-decarboxylase gene; *P_{Gal1}*, galactokinase promoter; *P_{T7}*, T7 RNA polymerase promoter.

a) pDH01



The *Herpes simplex* thymidine kinase (TK) gene from plasmid pJM81 (McNeil and Friesen, 1981) was amplified by the PCR method using primers that added EcoRI and SalI sites (5' GCTAGAATTTCGAGTATGGCTTCGTACC 3', 5' GCTAGTCGACCGTGTTTCATTAGCCTCC 3', sites underlined) upstream and downstream of the coding region, respectively. The TK gene was substituted for the ubiquitin gene cassette found in the yeast expression plasmid YEp96 (Ellison and Hochstrasser, 1991), such that the start and stop codons of each gene occupied identical positions with respect to the invariant non-coding flanking sequences that constitute the *CUP1* promoter and *CYC1* transcriptional terminator of YEp96. YEp96 is a high copy yeast/*E. coli* shuttle plasmid carrying the *TRP1* auxotrophic selectable marker.

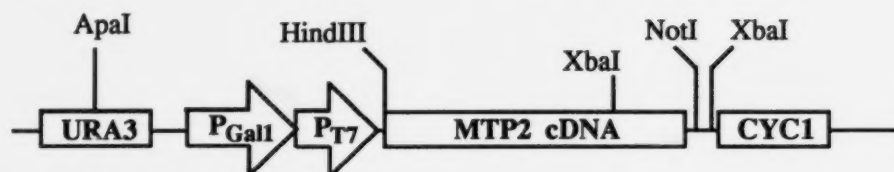
b) pMTP1



The plasmid pMTP1 was isolated during the functional complementation screen of a L1210/C2 cDNA library in yeast. The library vector was pYES2, a high copy

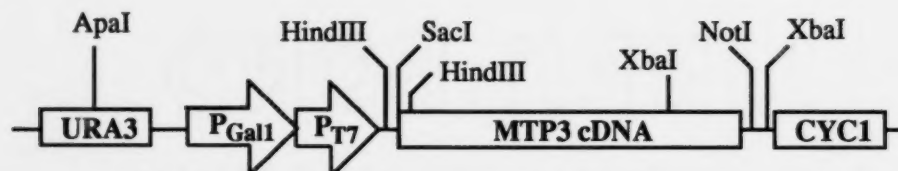
yeast/*E. coli* shuttle plasmid carrying the *URA3* auxotrophic selectable marker, *Gall* and T7 promoters, and the *CYC1* transcriptional terminator. The L1210/C2 cDNA library was constructed using cDNA that had been bi-directionally cloned into the *Bst*XI sites of pYES2. The *MTP1* cDNA encompassed nucleotides 384-1435 of the *MTP3* cDNA sequence shown in Fig. III 8.

c) pMTP2



An additional 348 nucleotides of *MTP* cDNA were identified and isolated from the L1210/C2 cDNA plasmid library by the anchor-PCR method using primers that corresponded to the *CYC1* terminator region of pYES2 (5' GCGTGAATGTAAGCGTG 3') and an internal region of the *MTP1* cDNA (5' CTTGCTTAATCTAGATTGTC 3', *Xba*I site underlined). The PCR fragment was ligated as a 1065-bp (5') *Xba*I-*MTP* cDNA-*Xba*I (3') fragment into the pT7-6 vector (Tabor, 1990) to yield the pT7-6-*MTP* plasmid. DNA sequencing of the PCR insert of pT7-6-*MTP* revealed the insert to contain an overlapping region corresponding to nucleotides 1-667 of the *MTP1* cDNA, as well as an additional 349 nucleotides of 5' cDNA sequence. The *MTP* cDNA fragment of pT7-6-*MTP* was then subcloned as a (5') *Hind*III-*Xba*I (3') fragment into the corresponding sites of the pMTP1 vector. This cloning strategy required that the *MTP1* cDNA region first be subcloned, as a *Bam*HI-*Xho*I fragment, into pBlueScript SK⁺ (pBS-*MTP1*) to generate a unique *Xba*I site. pBS-*MTP1* was digested with *Sac*I and *Xba*I to remove part of the cDNA region. The (5') *Sac*I-*MTP* cDNA-*Xba*I (3') fragment from pT7-6-*MTP* was then cloned into the respective sites of the digested pBS-*MTP1* to generate pBS-*MTP2*. The (5') *Hind*III-*MTP* cDNA-*Xho*I (3') region of pBS-*MTP2* was then cloned into the respective sites in pYES2. The pMTP2 vector encompassed nucleotides 88-1435 of the *MTP* cDNA sequence shown in Fig. III 8.

d) pMTP3



The extreme 5' region of the *MTP* cDNA was rescued from a λGT11 mouse brain cDNA library by the anchor-PCR method using the λGT11 right primer (5' GGAAGGATCCTGGCGAGGACTCCTGGAGCCCC 3') and a primer that corresponded to an internal region of the *MTP* cDNA (5' CCTGACAAAAGCTTTCGGCCC 3', *Hind*III site underlined). The ≈110 bp PCR product was cloned into pBlueScript SK⁺ and the DNA sequence of the PCR insert and flanking regions (≈100 bp) was determined. The PCR insert exhibited an overlapping sequence corresponding to nucleotides 1-59 of the cDNA insert of pT7-6-*MTP* and an additional 35 nucleotides of 5' cDNA sequence. The region encompassing nucleotides 1-87 was then amplified, using a primer that encompassed the 5' terminus of the *MTP* cDNA (5' AGACTGATGAGCTCAGGGCCC

GGGCCGAGGGC 3', SacI site underlined) and a pBlueScript SK⁺ primer (5' GTAAAA CGAGGCCAGT 3'), and cloned as a 93-bp (5') SacI-HindIII (3') fragment into the respective sites of pBS-MTP2. The resulting *MTP3* cDNA region, encompassing the entire 1435 nucleotide cDNA sequence shown in Fig. III 8, was cloned as a (5') SacI-XhoI (3') fragment into pYES2.

RESULTS

A Basis for Identification of Nucleoside Transporter Proteins by Functional Expression Cloning in *S. cerevisiae*

Two prerequisites for the identification of a cDNA-encoded NT protein by functional expression are (i) the absence of a protein with similar function in the plasma membrane of the cell type to be used as the expression host, and (ii) a simple and unambiguous method for identification and isolation of cells that express a functional recombinant NT protein.

S. cerevisiae lacks a salvage pathway for utilization of extracellular thymidine and relies on the thymidylate synthase-mediated methylation of dUMP to create dTMP (For review, see Jones and Fink, 1982). The complete inhibition of thymidylate synthase in yeast leads to depletion of intracellular dTMP and subsequent cell death, even when yeast are grown in the presence of exogenous thymidine (Little and Haynes, 1979). In particular, *S. cerevisiae* lacks two necessary components of the thymidine salvage pathway: a transport process that mediates the uptake of thymidine (Grenson, 1969) and a thymidine kinase to convert intracellular thymidine to dTMP (Grivell and Jackson, 1968).

The initial prerequisite for a functional expression cloning approach using *S. cerevisiae* appeared to be satisfied, since the above observations suggested that this yeast lacks a plasma membrane transport process that mediates the influx of thymidine. Thus, a yeast cell that expressed a functional mammalian NT protein in its plasma membrane should survive inhibition of *de novo* dTMP synthesis and form a colony in the presence of exogenous thymidine, provided an intracellular thymidine kinase (TK) was also present. Before attempting to isolate cDNAs encoding NT proteins by expression cloning in *S. cerevisiae*, it was necessary to (i) confirm the absence of mediated transport of thymidine and (ii) develop a strain of TK-expressing yeast that could be used as the host expression cell.

Characterization of Nucleoside Transport Processes Present in *S. cerevisiae*

A single nucleoside transport process capable of selectively mediating the influx of uridine has been reported in *S. cerevisiae* (Grenson, 1969). To confirm the presence of this transport process and to determine whether pyrimidine or purine nucleosides are potential permeants for other plasma membrane transport processes, the uptake of [³H]nucleosides over an extended period of time was examined. The cellular uptake of 100 μ M concentrations of thymidine, uridine, and adenosine by the *S. cerevisiae* strain KY114 is shown in Panel A of Fig. III 1. While KY114 cells appeared to be impermeable to thymidine and adenosine in uptake assays performed over a 60-min period, uridine was readily taken up by the cells. These results confirmed the reported absence of any mediated influx of thymidine in *S. cerevisiae* and also demonstrated the absence of adenosine influx, at least at concentrations $\leq$ 100 μ M.

The uptake of uridine by KY114 cells may have occurred by either mediated transport and/or passive diffusion. The presence of a mediated transport process can often be established by the ability of an excess of non-radioactive permeant to inhibit the influx of the radioactive tracer permeant. Panel B of Figure III 1 shows the results of an assay that examined the uptake of 100 μ M [³H]uridine in the absence and presence of 10

mM non-radioactive uridine. A 100-fold excess of uridine almost completely inhibited [^3H]uridine uptake, suggesting uridine uptake occurred via a mediated process(es) in KY114 cells.

NBMMPR and dipyridamole are capable of inhibiting mammalian equilibrative NT processes when present at nanomolar concentrations. To assess whether uridine transport in *S. cerevisiae* was sensitive to inhibition by these compounds, the uptake assays (Fig. III 1) were also performed in the presence of 10 μM concentrations of NBMMPR (Panel A) or dipyridamole (Panel B). Neither NBMMPR or dipyridamole significantly inhibited the uptake of 100 μM uridine into KY114 cells, which indicated that the transport process(es) responsible were insensitive to these compounds.

The permeant selectivity of the uridine transport process(es) was examined in greater detail by measuring uptake of 100 μM [^3H]uridine in the presence of 10 mM concentrations of structurally related pyrimidine nucleosides, uracil, and purine nucleosides (Fig. III 2). Inhibition of [^3H]uridine uptake was negligible in assays conducted in the presence of 10 mM adenosine, inosine, thymidine, 5-fluoro-2'-deoxyuridine, cytidine, and deazauridine. Uridine uptake was inhibited by 50% or less in the presence of pseudouridine and 5'-fluorouridine and was inhibited approximately 50% in the presence of 2'-deoxyuridine and uracil. These observations suggested this "uridine-selective" influx process would not mediate the influx of thymidine, thereby confirming absence of any mediated influx of thymidine in *S. cerevisiae*.

Generation of a Yeast Strain for Use in an Expression Cloning Procedure

Before using *S. cerevisiae* as an expression host cell in a selection medium based on the salvage of extracellular thymidine, it was necessary to generate a yeast strain that expressed a functional thymidine kinase. The *Herpes simplex* TK gene had previously been expressed in *S. cerevisiae* using high-copy *E. coli*/yeast expression vectors (McNeil and Friesen, 1981; Zhu *et al.*, 1984), suggesting that it should be possible to create a TK-expressing yeast strain that would be suitable for use as the expression host cell.

The *H. simplex* TK gene was amplified by the polymerase chain reaction (PCR) method and inserted behind the *CUP1* promoter in the yeast expression plasmid YEp96, as described in Methods in Materials. The YEp96 vector contains the *TRP1* auxotrophic selectable marker, which provides for maintenance of the plasmid in the *trp1* auxotrophic KY114 yeast strain. The *CUP1* promoter mediates a constitutive low level transcription, however transcription can be greatly increased in the presence of Cu^{2+} . The resulting vector pDH01 was transformed into KY114 cells to create the KTK yeast strain, which was found to be capable of expressing the TK gene product in a copper-inducible manner using an assay for TK activity in cell extracts.

Cell extracts were prepared from KY114 and KTK cells that had been grown in the absence or presence of Cu^{2+} and were examined for their capacity to form [^3H]dTMP by phosphorylation of [^3H]thymidine. The results of these assays are shown in Fig. III 3. Cell extracts from KY114 cells, grown either in the absence or presence of Cu^{2+} , failed to produce [^3H]dTMP over a period of 90 min. However, a substantial level of [^3H]dTMP formation over the same time period was observed in cell extracts prepared from KTK cells grown in the absence of Cu^{2+} . An approximate 15-fold increase in levels of [^3H]dTMP generation was observed in assays conducted with cell extracts prepared from KTK cells grown in the presence of Cu^{2+} . These observations confirmed (i) the absence of thymidine kinase in KY114 cells, (ii) the presence of low-level constitutive expression of thymidine kinase by KTK cells in the absence of Cu^{2+} , and (iii) that the expression of the thymidine kinase gene in KTK cells was under control of the Cu^{2+} -inducible *CUP1* promoter of the pDH01 vector.

High concentrations of dTTP has previously been demonstrated to inhibit both the bacterial (O'Donovan, 1978) and *H. simplex* thymidine kinases (McNeil and Freisen,

1981). To confirm the substrate selectivity of the *H. simplex* thymidine kinase expressed by KTK cells, thymidine kinase assays were conducted in the presence of 250 mM concentrations of dTTP, 2'-deoxyuridine, and 2'-deoxycytidine (Fig. III 3). A substantial inhibition of thymidine kinase activity was observed in the presence of dTTP and 2'-deoxyuridine, whereas little inhibition was noted in the presence of 2'-deoxycytidine. These results confirmed the characteristic inhibition of *H. simplex* thymidine kinase by dTTP and provided evidence for the substrate selectivity of the recombinant thymidine kinase.

The characteristics of the KTK yeast strain suggested it could be used as the host cell type identification of cDNAs encoding NT proteins in an expression cloning procedure.

Determination of Conditions for Selection of Yeast Expressing NT Proteins

The identification of KTK cells that expressed a cDNA-encoded protein capable of mediating the influx of thymidine required selection of conditions under which (i) the complete inhibition of thymidylate synthase was achieved and (ii) passive diffusion of exogenous thymidine into KTK cells was negligible. These conditions were determined in a series of experiments in which the TK-negative (KY114) and TK-expressing (KTK) cells were exposed to inhibitors of dTMP synthesis in the presence or absence of exogenous thymidine.

Since the thymidylate synthase of *S. cerevisiae* has previously been shown to be completely inhibited by the combined action of sulfanilimide and methotrexate (Little and Haynes, 1979; McNeil and Freisen, 1981), these drugs were used to block the production of dTMP. The survival of KY114 and KTK cells plated onto medium that contained sulfanilimide and methotrexate, as well as increasing various concentrations (0-4000 μ M) thymidine was examined. It was observed that KY114 cells could not survive on this medium regardless of the concentration of thymidine present. However, KTK cells were able to survive and form colonies in the presence of ≥ 500 μ M thymidine. The results of one such plating assay are shown in Fig. III 4. KTK cells were unable to survive and form colonies in the absence of thymidine, suggesting that sulfanilimide and methotrexate had fully inhibited thymidylate synthase. Although KTK cells were also unable to form colonies in the presence of 100 μ M thymidine, they survived in the presence of 1000 μ M thymidine. These results suggested that a high exogenous concentration (1 mM) of thymidine was sufficient to rescue KTK cells, presumably by passive diffusion and subsequent phosphorylation of thymidine by *H. simplex* thymidine kinase. Because KY114 cells lack a thymidine kinase, they could not be rescued with thymidine when exposed to methotrexate and sulfanilimide under identical conditions.

The results of Fig. III 4 suggested that a KTK yeast cell which expressed a functional recombinant NT protein should be able to survive in the presence of methotrexate, sulfanilimide, and diffusion-limited concentrations of thymidine, since influx of thymidine would be made possible by the recombinant NT protein. During the experiments described above, it was observed that 5 μ M methotrexate and 2 mg/ml sulfanilimide were sufficient to inhibit thymidylate synthase. However, the expression screening method was performed using higher concentrations of these drugs to minimize the possibility of generating KTK cells that may acquire resistance to the effects of the drugs.

Isolation of a Partial Mouse Transporter Protein (MTP1) cDNA That Functionally Complements in the Yeast Expression Cloning Approach

The aim of this study was to identify cDNAs that encode equilibrative NT proteins. Since the *es* and *ei* NT processes of L1210/C2 cells accept thymidine as a permeant and have been well characterized (Belt, 1983; Dagnino, 1988; Hogue *et al.*, 1990), this cell type was used as a source of mRNA for the construction of an expression library. A cDNA yeast expression library was constructed in the high copy pYES2

yeast/*E. coli* shuttle vector by Invitrogen Corporation using polyA⁺ RNA isolated from L1210/C2 cells. The pYES2 vector contains the *URA3* auxotrophic selectable marker and a multiple cloning site situated downstream of the galactose-inducible (glucose-repressible) *Gall* promoter and upstream of the *CYC1* transcriptional terminator. The *URA3* selectable marker allows for maintenance of the plasmid in a *ura3* auxotrophic yeast strain that is grown in the absence of uracil. The *Gall* promoter provides a high-level of transcription of cDNA insert in the presence of galactose and absence of glucose. The cDNA was generated from L1210/C2 polyA⁺ RNA using Avian Myeloblastosis Virus reverse transcriptase. Blunt-ended cDNA was ligated to non-palindromic BstXI linkers and the linkered cDNA (≥ 1000 bp in length) were inserted into the pYES2 vector.

Two independent groups of yeast transformants were generated by electroporation of KTK yeast cells with the L1210/C2 pYES2 expression library on separate occasions. These transformants were grown in the presence of glucose to avoid the expression of cDNA-encoded proteins which may induce decreased levels of cell growth, and hence be under-represented in the yeast "cDNA library". In each case, electroporation mixtures were plated onto 2% glucose/CMM that lacked uracil and tryptophan and transformant colonies obtained were then washed off the plates with 2% glucose/CMM and independently subjected to the following functional expression screening protocol.

The first group of transformants (1.0×10^5 independent transformants) was plated onto 2% galactose/CMM that contained methotrexate (125 $\mu\text{g/ml}$), sulfanilimide (6 mg/ml) and 75 μM thymidine and yielded 300 colonies after incubation for 3 days at 30°C. The second group of transformants (0.75×10^5 independent transformants), which was plated onto 2% galactose/CMM that contained methotrexate (25 $\mu\text{g/ml}$), sulfanilimide (6 mg/ml) and 250 μM thymidine, yielded 122 colonies. For each group of transformants, one streak of each colony was placed onto (i) 2% glucose/CMM plates that lacked tryptophan and contained fluoroorotic acid (FOA; 0.5 mg/ml) and uracil or (ii) 2% glucose/CMM plates that lacked tryptophan and uracil. Glucose was substituted for galactose in this media because (i) yeast grow more quickly in a glucose-containing medium and (ii) this step involved the selection for and against the *URA3* selectable marker of the library plasmid and did not require the expression of the cDNA insert. Colonies that arose on the FOA-containing plates should have lost the library plasmid since the presence of the *URA3* selectable marker (orotidine-5'-P-decarboxylase gene) confers sensitivity to FOA toxicity. Conversely, colonies that arose on the FOA-free plates that lacked tryptophan and uracil must have retained both the library plasmid and pDH01 in order to survive. Colony isolates that failed to grow on plates lacking tryptophan and uracil were discarded since these isolates must have lacked one (or both) vectors and were, therefore, false positives. All of the isolates that grew on both types of plates were then subjected to an additional round of screening.

The second round of screening involved the streaking of the FOA-selected and non-FOA-selected cells of a single isolate, side by side, onto 2% galactose/CMM plates that contained the concentrations of methotrexate, sulfanilimide, and thymidine used in the initial selection medium. Growth of the FOA-selected isolates on these plates confirmed that these were "false positive", since no library plasmid should have been present in these cells. Thus, a colony isolate was deemed to contain a positive cDNA clone when it exhibited (i) no growth of FOA-selected cells and (ii) growth by cells selected on plates that contained uracil and tryptophan.

A total of 12 independent positive isolates (10 clones in first group of transformants, 2 clones in the second group of transformants) were isolated and the plasmid DNA isolated from each was transformed into *E. coli*. Plasmid mini-preps were prepared from bacterial transformants and the cDNA-containing pYES2 vectors were identified on the basis of restriction enzyme digestion analysis. To confirm that the cDNA-encoded protein conferred thymidine uptake in KTK cells, these cells were transformed with the isolated cDNA library plasmids and plated onto 2% galactose/CMM

plates that contained the concentrations of methotrexate and sulfanilimide used in the initial selection medium and either (i) no thymidine or (ii) 100 μ M thymidine. The ability of all transformants to grow in the presence of thymidine, but not in its absence, confirmed that thymidine uptake had occurred. The library plasmids were digested with HindIII and XbaI to excise the cDNA inserts and all were found to yield identical restriction fragments of $\approx$ 400 and 650 bps. Since the library plasmids appeared to contain the same $\approx$ 1.0 kbp cDNA insert, one of the cDNA-containing plasmids designated pMTP1 (Mouse Transporter Protein 1) was selected for further study.

The functional expression of pMTP1 under the conditions of the expression screening procedure is shown in Fig. III 5. KTK cells transformed with pMTP1 were able to survive under conditions of thymidylate synthase inhibition in the presence of 100 μ M thymidine, but not in the absence of thymidine. Under identical conditions, KTK cells transformed with pYES2 failed to survive in the presence of 100 μ M thymidine. These results suggested that the *MTP1* protein mediated thymidine uptake in KTK cells. The yeast plasma membrane is normally impermeable to dTMP (Little and Haynes, 1979; McNeil and Freisen, 1981), however should dTMP enter the cell it would be expected to confer cell survival. To assess whether the *MTP1* protein mediated dTMP flux, pMTP1-transformed KTK cells were plated onto medium that contained methotrexate, sulfanilimide, and 100 μ M dTMP (in place of thymidine). No cell survival was observed, suggesting that the *MTP1* cDNA encoded a protein that selectively mediated the uptake of thymidine, but not dTMP.

In addition, pMTP1-transformed KTK cells were plated onto medium that contained methotrexate, sulfanilimide, 100 μ M thymidine and either NBMPR or dipyridamole (Fig. III 6). KTK cells transformed with pMTP1 were able to grow equally well in the absence and presence of 25 μ M NBMPR or 10 μ M dipyridamole, suggesting that the process which mediated the uptake of thymidine was insensitive to inhibition by these inhibitors.

The nucleotide sequence of the 1052 bp *MTP1* cDNA was determined and was found to contain a polyadenylation consensus signal sequence and polyadenylated 3' terminus. This cDNA contained an open reading frame from its 5' terminus, therefore the possibility existed that the *MTP1* cDNA represented only a 3' region of a larger cDNA. Northern blot analyses were performed against identical quantities of RNA isolated from various sources to confirm whether the *MTP1* cDNA represented a "full length" clone and to determine the extent and distribution of *MTP* mRNA. As illustrated in Panel A of Fig. III 7, the *MTP1* cDNA probe hybridized to L1210/C2 RNA of approximately 1500 nucleotides in size, as well as to a smaller RNA species. This result suggested that the 1052 nucleotide *MTP1* cDNA most probably represented a partial *MTP* RNA transcript. A hybridization signal of similar strength was also observed in RNA of L1210/B23 and L1210/MA27 cells, human HeLa cells and rat W256 cells. The signal was much weaker in RNA from human HL60 cells and was completely absent in RNA isolated from *S. cerevisiae* cells and mouse lymphoma S49 and AE₁ cells. A hybridization signal of similar size was observed in RNA isolated from the brain, kidney, liver, and spleen of mouse, as shown in Panel B of Fig. III 7. The signal was of highest intensity in kidney, of moderate intensity in brain and spleen and of very low intensity in liver. The distribution of the 1500 nt hybridization signal suggested that (i) *MTP* RNA transcript is widely expressed in most tissues and cell types, albeit at differing levels and (ii) the *MTP1* cDNA constituted a partial cDNA clone.

In an effort to identify potentially longer *MTP* cDNAs, the L1210/C2 cDNA plasmid library and a λ GT11 mouse brain cDNA library (John Gardner, Fox Chase Cancer Center) were screened by anchor-PCR methodology. This approach employed primers that corresponded to regions of (i) pYES2 and λ GT11 which flanked the cDNA insertion site and (ii) the *MTP* cDNA that exhibited unique restriction sites. Additional 5' regions of cDNA were isolated from both cDNA libraries and were cloned in-frame with

the *MTP1* cDNA, as described in detail in the Construction of Expression Vectors section of Methods and Materials. The result was a putative full length *MTP* cDNA of 1435 nucleotides which was designated *MTP3*.

Characterization of the Full Length *MTP* cDNA Clone

The nucleotide and predicted amino acid sequences of the putative full length *MTP3* cDNA are shown in Fig. III 8. The assignment of the first ATG codon as a translational start site predicted (i) the presence of a putative 5' untranslated region of 135 nucleotides, (ii) an open reading frame (ORF) of 657 nucleotides that terminated with a TGA codon, and (iii) a 3' untranslated region of 643 nucleotides. The predicted translation initiation codon and its flanking sequence (5' TTCCGGATGG 3') did not generally resemble the consensus sequence for translation initiation in eucaryotic cells (5' GGA/GCCATGG 3'; Kozak, 1991). The 3' untranslated region contained (i) a polyadenylation consensus signal sequence (5' AATAAA 3'; Birnstiel *et al.*, 1985) at a position 13 nucleotides upstream of the polyadenylated tail and (ii) two 5' ATTTA 3' sequence motifs previously demonstrated to be signals for selective mRNA degradation (Shaw and Kamen, 1986).

The ORF of the *MTP3* cDNA encoded a 219 amino acid protein with a predicted molecular weight of 25.2 kDa. An examination of the amino acid sequence of the *MTP3*-encoded protein revealed the presence of a single potential N-linked glycosylation consensus sequence at Asn 189 (Asn-Thr-Ser). Consensus phosphorylation sequences for cAMP kinase, casein kinase II, and protein kinase C were found to reside within 30 residues of the amino terminus of the *MTP3*-encoded protein (Hart, 1992; Kenelly and Krebs, 1991).

The relative hydrophobicity of the *MTP3*-encoded protein was assessed using the Kyte-Doolittle algorithm (Kyte and Doolittle, 1982), the results of which are shown in Fig. III 9. The *MTP3*-encoded protein exhibited hydrophilic regions of about 20 amino acid residues at the carboxyl and amino termini and was predicted to contain 4 distinct regions of hydrophobicity, each encompassing approximately 25 amino acids. The hydropathic profile was consistent with the presence of 4 potential membrane-spanning domains and allowed for the classification of the *MTP3*-encoded protein, according to the nomenclature of Singer (1990), as a polytopic type III integral membrane protein.

The cDNA of the pMTP1 plasmid originally isolated in the functional screening procedure encompassed amino acid residues 84-219 of the predicted *MTP3*-encoded protein shown in Fig. III 8. Assuming that the ATG codon at nucleotide 403 of the *MTP3* cDNA sequence could function as a translation initiation codon, the *MTP1* cDNA was predicted to encode a 15.2-kDa protein product. The actual sizes of the proteins encoded by the *MTP1* and *MTP3* cDNAs were determined directly by an *in vitro* transcription/translation technique. RNA transcripts were produced from pMTP1 and pMTP3 by transcription from the T7 RNA polymerase promoter of the pYES2 vector, then translated in rabbit reticulocyte lysate in the presence of ³⁵S-methionine. A control translation reaction was performed in the absence of template RNA. The ³⁵S-labeled protein was resolved by SDS-PAGE and visualized by autoradiography, as shown in Fig. III 10. Translation of the *MTP1* and *MTP3* RNA transcripts gave rise to 16 and 23.5 kDa protein products, respectively, that were absent in the control reactions. These results confirmed that proteins of the predicted molecular weights were not only encoded by, but could also be expressed from, the ORFs of the *MTP1* and *MTP3* cDNAs. However, the observation that *in vitro* translation of these RNA transcripts repeatedly gave rise to weak signals for their respective ³⁵S-labeled proteins suggested that the rabbit reticulocyte lysate was inefficient at initiating translation from these transcripts³.

³ This statement is based upon the results observed in three separate *in vitro* transcription/translation experiments.

The *MTP* cDNA Encodes a Novel Protein

To determine the relatedness of the *MTP* to other identified proteins, the ORF nucleotide sequence of the *MTP3* cDNA (Fig. III 8) and its predicted protein sequence were compared to the nucleotide and protein databases of the NCBI and GCG packages, as described in Methods and Materials. Only a single nucleotide entry, designated *HUMORF13* (Accession D14696, GenBank nucleotide database), was found to exhibit any identity with the ORF of the *MTP3* cDNA. *HUMORF13* is a 1402-bp cDNA isolated from human myeloblast KG-1 cells that contains an ORF of 699 nucleotides (Momura *et al.*, 1993). An alignment of the ORFs of the *MTP3* and *HUMORF13* cDNA sequences is presented in Fig. III 11. The two nucleotide sequences exhibited a 92% identity. A similar alignment of their predicted protein products revealed that the *MTP3* and *HUMORF13* protein sequences shared a 97% identity and 98% similarity at the amino acid level. It was apparent from these results that *HUMORF13* represents a human homolog of the *MTP* protein. The 5' and 3' untranslated regions of the *HUMORF13* and *MTP3* nucleotide sequences were found to exhibit 75-80% identity. No other database entries exhibited nucleotide or peptide sequence identity to either localized regions or to the overall sequence of *MTP*, suggesting that *MTP* and its human homolog *HUMORF13* represent members of a novel protein family.

Identification of the Promoter Region of the *MTP* Gene

In an effort to identify the promoter region of the *MTP* gene, a mouse λ FIX II genomic DNA library was hybridization screened with a *MTP1* cDNA probe. Positive phage clones were isolated and subjected to a second round of hybridization screening using an oligonucleotide probe (5' CCGGTGCGGACATGGAAACAG 3') that corresponded to sequence in the 5' untranslated region of the *MTP3* cDNA sequence shown in Fig. III 8. A single clone that contained a ~20 kbp genomic insert was isolated and a 703-bp HindIII fragment of this insert, capable of hybridizing to the oligonucleotide probe, was subcloned and its DNA was sequenced.

The sequence of the 610-bp genomic DNA region that resides 5' upstream of the deduced *MTP3* cDNA sequence, together with the first 162 nucleotides of the cDNA sequence, is shown in Fig. III 12. The genomic sequence, presumably representing at least part of the promoter region for the *MTP* gene, did not contain a TATAA transcription initiation sequence motif such as are present in the promoter regions of many mammalian genes (Ghosh *et al.*, 1981; Blake *et al.*, 1990). However, the *MTP* promoter region did exhibit a motif (CCAAT) that has been implicated in the regulation of transcription efficiency (Jones *et al.*, 1987; Chodosh *et al.*, 1988; Blake *et al.*, 1990). Multiple CpG islands (GGCC or CCGG) were found to be present in areas that flanked the 5' terminus of the *MTP3* cDNA (bp 541-744 of Fig. III 12). A G/C box (GGGCGG) that constitutes a decanucleotide consensus sequence for the binding of Sp1 transcription factor (Kadonga *et al.*, 1986) was also present within the 5' untranslated region of the *MTP* cDNA sequence. The presence of CgG islands and G/C boxes within TATAA-less promoters (or within the 5' untranslated regions of genes with TATAA-less promoters) has been observed primarily in housekeeping genes, a class of genes expressed at low levels in almost every tissue and at every stage of development (Gardiner-Garden and Frommer, 1987).

The sequence of the *MTP* promoter region was also compared to a compilation of DNA binding sequences for vertebrate transcription factors (Faissst and Meyer, 1992). Binding sites of perfect consensus DNA sequence were observed for several transcription factors, including NF-IL6 (TGNNGNAAG; Akira *et al.*, 1990), CF1 (ANATGG; Riggs *et al.*, 1991), PEA3 (TTTCCT; Wasylyk *et al.*, 1989), LyF-1 (PPTGGGAGR; Lo *et al.*, 1991), and HIP1 (ATTCN₍₁₋₃₀₎GCCA; Means and Farnham, 1990). The HIP1 binding sequence has only been observed in the TATAA-less promoters of housekeeping genes (Means and Farnham, 1990). Results of this sequence analysis of the *MTP* promoter

region, combined with the presence of CgG islands and the G/C box in the TATAA-less promoter, suggested that the *MTP* gene belonged to the class of housekeeping genes.

DISCUSSION

The nucleoside uptake experiments conducted during this study supported previous observations (Jones and Fink, 1982; Grenson, 1969) that although *S. cerevisiae* lack the capacity for mediated uptake of thymidine and adenosine, they are capable of accumulating [³H]uridine. The almost complete inhibition of [³H]uridine uptake in the presence of a large extracellular concentration of non-radioactive uridine suggested that uptake had occurred via a mediated transport process(es). A strict pyrimidine nucleoside selectivity for this transport process(es) was suggested by the results of [³H]uridine uptake assays conducted in the presence of a 100-fold excess concentration of potential competitors (Fig. III 2). The 2'-hydroxyl group and C5 positions were evidently important structural features for permeant recognition since uridine uptake was inhibited ≈50% in the presence of high concentrations of pseudouridine, 5-fluorouridine and 2'-deoxyuridine. The inability of purine nucleosides, cytidine, thymidine, 5-fluoro-2'-deoxyuridine or deazauridine to inhibit uridine uptake suggests that the transport process is selective for pyrimidine nucleosides and does not accept structural analogs of uridine that are altered at the N3 position in the pyrimidine ring or a combination of a methyl group at the C5 position and lack of 2'-hydroxyl group. Since a high concentration of 2'-deoxyuridine or uracil inhibited ≈ 50% of uridine uptake, the 2'-hydroxyl group may also be required for permeant recognition.

The lack of inhibition of uridine uptake in *S. cerevisiae* by NBMPR is consistent with the inability of the transport process(es) to accept purine nucleosides as permeants. Dipyridamole is a non-nucleoside inhibitor of mammalian equilibrative NT processes analog and was also unable to inhibit uridine transport in this yeast.

NT processes of mammalian and most non-mammalian eucaryotic cells⁴ identified to date accept both adenosine and uridine as permeants. An exception is *Spodoptera frugiperda* Sf9 cells which express distinct purine and pyrimidine nucleoside NT processes (Hogue and Cass, 1994). It appears, therefore, that the uridine transport process of *S. cerevisiae* is an example of an extreme exception to the permeant selectivity of NT processes in general.

The proposed expression cloning method was based upon the observation that *S. cerevisiae* (i) rely on the thymidylate synthase-mediated methylation of dUMP to create dTMP and (ii) lack a salvage pathway for utilization of extracellular thymidine, which normally consists of a thymidine influx process and TK. It was hypothesized that under conditions of thymidylate synthase inhibition, TK-expressing yeast cells would undergo dTMP depletion unless a recombinant NT process was made available to mediate influx of exogenous thymidine. Thus, cell survival under these conditions would provide a means to identify cells expressing a cDNA-encoded NT protein.

This cloning method required the generation of a strain of *S. cerevisiae* capable of expressing TK. The KTK yeast strain, which contained the *H. simplex* TK gene on the YE96 plasmid, was shown capable of expressing low levels of TK activity in a constitutive manner from the *CUP1* promoter. Exposure of KTK cells to CuSO₄ resulted in an ≈15-fold increase in TK activity, a level of induction with this promoter similar to that observed in other applications (Romanos *et al.*, 1992). Plating studies revealed that

⁴ A review of NT processes exhibited by non-mammalian eucaryotic cells is presented in Chapter V of this thesis.

the low constitutive expression of TK activity was sufficient to mediate survival of thymidylate synthase-inhibited KTK cells in the presence of 1.0 mM, but not 100 μ M exogenous thymidine (Fig. III 4). It appeared that a high exogenous concentration (≥ 0.5 mM) of thymidine was sufficient to rescue KTK cells, presumably by passive diffusion. These results suggested that in the presence of <500 μ M thymidine and under conditions of thymidylate synthase inhibition, only KTK cells that expressed a recombinant NT protein should survive.

The *es* and *ei* NT processes of L1210 cells have been well characterized and accept thymidine as a permeant. Thus, L1210/C2 cells were chosen as a source of polyA⁺ RNA to construct a mammalian cDNA expression library. The KTK yeast strain was transformed with a L1210/C2 cDNA expression library on two independent occasions and subjected to the expression cloning method. An identical species of L1210/C2 cDNA clone (*MTP1*) was isolated in each case. Plating assays conducted under thymidylate synthase-inhibiting conditions revealed that the presence of thymidine, but not dTMP, led to cell survival. This suggested the cDNA-encoded protein mediated influx of nucleosides but not nucleotides. The activity of the *MTP1* cDNA-encoded protein was insensitive to inhibition by NBMPR and dipyridamole, which normally inhibit mammalian equilibrative NT processes at micromolar concentrations.

The *MTP1* cDNA clone proved to be a partial clone that lacked a 5' region of untranslated and ORF DNA sequence. The missing 5' region was isolated from (i) a longer *MTP2* cDNA insert of the L1201/C2 cDNA library and (ii) a λ GT11 mouse brain cDNA to obtain the putative full length *MTP3* cDNA shown in Fig. III 6. The ORF of *MTP3* cDNA predicts a 25.2 kDa polytopic protein with four hydrophobic potentially membrane-spanning domains. The ORF of *MTP1* cDNA predicts a 15.2 kDa protein that includes most of the second and all of the third and fourth hydrophobic domains. *In vitro* transcribed *MTP3* and *MTP1* cRNAs gave rise to 23.5 and 16 kDa proteins, respectively, upon translation in rabbit reticulocyte lysates. These values closely agree with the predicted molecular weights, despite the sometimes anomalous migration of hydrophobic proteins through SDS-polyacrylamide gels. *MTP* was originally identified in the expression cloning procedure as a cDNA-encoded truncated protein (*MTP1*). The capacity of the *MTP1*-encoded protein to rescue NT-deficient yeast from inhibition of thymidylate synthase was surprising, since it implies that the truncated protein was capable of mediating thymidine transport. Such a situation is not without precedence, since the lactose permease of *Escherichia coli* has been altered by deletion and insertion mutagenesis and shown to mediate lactose permeation, albeit at lower activities than the wild-type permease (McKenna *et al.*, 1992; Bibi and Kaback, 1992).

An analysis of the putative promoter region of the *MTP* gene revealed characteristics of a typical housekeeping gene promoter. These characteristics include the lack of a TATAA motif and the presence of CgG islands, a G/C box, a CCAAT motif and a HIP1 transcription factor binding sequence. The significance of the presence of the binding sites of consensus DNA sequence for the CF1, NF-IL6, PEA3 and LyF-1 transcription factors is unknown. Should this putative promoter region actually function as a housekeeping promoter, a low level of *MTP* expression may be generally observed in cells.

Northern blot analyses revealed the *MTP* RNA transcript to be expressed in a variety of mouse tissues and cultured mouse cell lines. Transcript closely related to that of *MTP* was expressed at varying levels in a variety of rat and human cell lines. As expected, *S. cerevisiae* lacked the transcript related to *MTP*. The pattern of *MTP* RNA distribution in cultured cell lines was not consistent with the expression of *es*, *ei*, or

concentrative NT processes⁵. *MTP* transcript was absent in S49 and AE₁ cells which express *es* NT and no NT, respectively (Cass *et. al.*, 1981). As well, *MTP* transcript was present in L1210/MA27 and Walker 256 cells, which do not express *es* NT (Crawford *et. al.*, 1990; Belt and Noel, 1985), suggesting that the *MTP* clone does not represent an *es*-type NT protein. The presence of *MTP* transcript in L1210/B23 (Vijayalakshmi *et. al.*, 1992) and L1210/MA27 cells, cell types that do not express *ei*-type NT, suggested the *MTP* clone does not represent an *ei*-type NT protein. *MTP* probably does not represent a concentrative NT protein, since it is expressed in cell lines besides only the L1210/C2 (Dagnino, 1988), L1210/MA27 and Walker 256 cell lines (Crawford and Belt, 1991) which do express a concentrative NT process.

A human homolog of *MTP* (*HUMORF13*) was identified by searching the GenBank database. No other proteins were found to exhibit a significant degree of identity with *MTP*, although the presence of a similar homolog in rat (Walker 256 cells) was suggested on the basis of Northern blot analysis. The predicted mouse and human amino acid sequences were 97% identical, an observation that suggested a highly-conserved functional nature for these 4 membrane-spanning domain proteins. To date, the majority of substrate transporter proteins identified have been classified into three general categories: (i) 12 membrane-spanning domain transporters (Griffith *et. al.*, 1992; Hebert and Carruthers, 1992; Marger and Saier, 1993), (ii) 6 membrane-spanning domain mitochondria transporters (Runswick *et. al.*, 1990) and major intrinsic protein (MIP) family (Reizer *et. al.*, 1993) and (iii) a small group of transporters exhibiting 6-10 membrane-spanning domains (Amara, 1992). It is apparent that *MTP* cannot be easily classified amongst these types; however its hydrophobic topography does bear resemblance to 4 membrane-spanning domain channel proteins such as the type I transmitter-gated ion channels (ie. glycine and acetylcholine receptors) (Barnard, 1992) and connexins, which act as gap junction channels between cells (Bennett *et. al.*, 1991). In addition, several bacterial proteins responsible for the cellular efflux of cytotoxic compounds (ethidium, methyl viologen) exhibit 4 membrane-spanning domains (Purewal, 1991; Morimyo *et. al.*, 1992; Sasatsu *et. al.*, 1992). The peroxisomal PMP 22 protein (Kaldi *et. al.*, 1993), the Mpv17 protein (Weiher *et. al.*, 1990) and the lens MP20 protein (Kumar *et. al.*, 1993; Gutskunst *et. al.*, 1990) also exhibit 4 membrane-spanning domains. Interestingly, the function of the PMP 22 and MP20 proteins appear to be related to peroxisome (Van Veldhoven *et. al.*, 1987; Lemmens *et. al.*, 1989) and lens fiber cell permeability, respectively. Gene inactivation of Mpv17 has been demonstrated to induce nephrotic syndrome and renal failure, however the true function of the ubiquitously expressed Mpv 17 protein is unknown (Weiher *et. al.*, 1990). These observations suggest that the 4 membrane-spanning domain proteins, including *MTP*, may generally confer permeability to small molecules.

⁵ The NT-deficient AE₁, L1210/MA27 and L1210/B23 cell lines were generated by frame-shift mutagenesis, therefore a possibility exists that these cell types may still express RNA transcript for the NT proteins that are not functionally expressed.

FIGURE III 1

***S. cerevisiae* Possess a Uridine Uptake Process and Lack the Capacity For Thymidine and Adenosine Uptake**

Actively proliferating *S. cerevisiae* grown in YPD were pelleted by centrifugation and suspended in replicate cultures ($OD_{600} = 0.80$) in 2% (w/v) glucose/CMM that contained 100 μ M [^{3} H]nucleoside alone or together with 10 μ M NT inhibitor. The cell/[^{3} H]nucleoside suspensions were incubated (shaking) at 30°C. The cell population doubling time in 2% (w/v) glucose/CMM under these conditions was estimated to be approximately 3 hr. At 15-min intervals, 200- μ l portions of cell suspensions were layered over oil in microcentrifuge tubes and subjected to centrifugation as detailed in Methods and Materials. All assays were conducted in triplicate and the radioactivity associated with the cell pellet was measured by liquid scintillation counting. Data are expressed as mean values $\pm$ SD; error bars are not shown where SD values were smaller than the size of data symbols.

Panel A. Uridine, but not thymidine and adenosine, is internalized by *S. cerevisiae*. Uptake of 100 μ M [^{3} H]uridine (O), [^{3} H]thymidine ($\square$), [^{3} H]adenosine (Δ) or 100 μ M [^{3} H]uridine in the presence of 10 μ M NBMPR ($\bullet$) was measured at the times indicated.

Panel B. Uridine uptake occurs by a dipyrindamole-insensitive mediated process. Uptake of 100 μ M [^{3} H]uridine was measured after suspension of cell pellet in 2% (w/v) glucose/CMM that contained (i) 100 μ M [^{3} H]uridine alone ($\bullet$), (ii) 100 μ M [^{3} H]uridine and 10 μ M dipyrindamole ($\square$) or (iii) 100 μ M [^{3} H]uridine and 10 mM non-radioactive uridine (O).

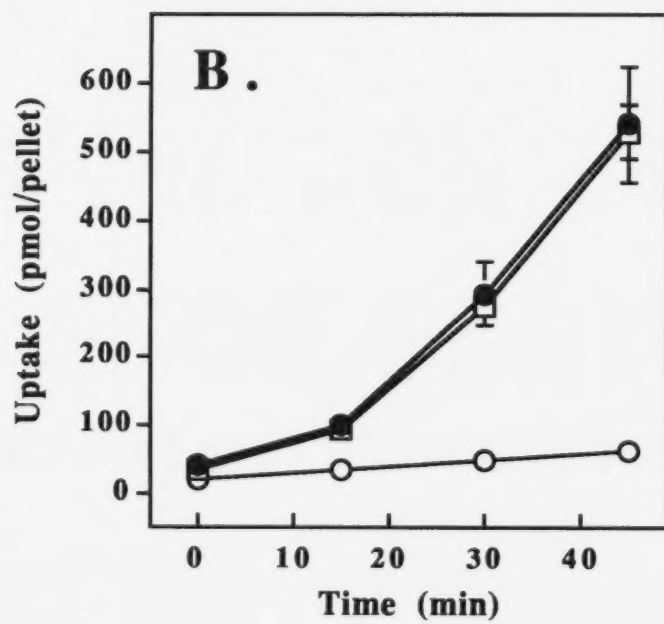
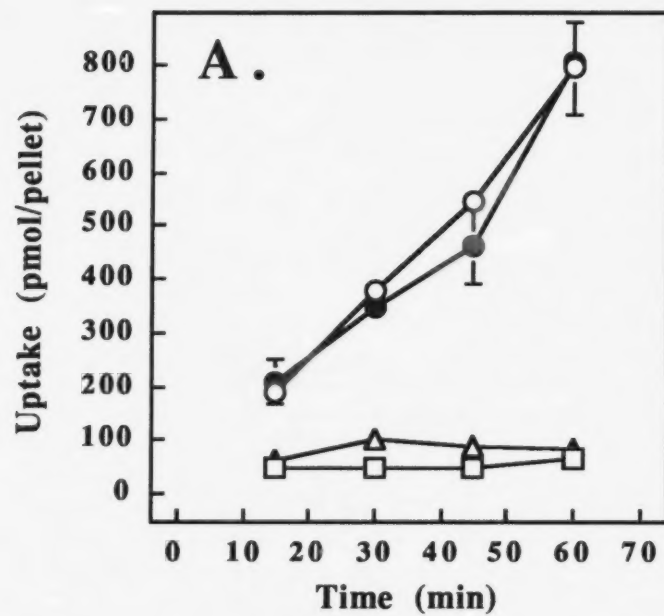


FIGURE III 2

Uridine Uptake is Mediated by a Highly-Selective Transport Process in *S. cerevisiae*

Actively proliferating *S. cerevisiae* grown in YPD were pelleted by centrifugation and suspended in replicate cultures ($OD_{600} = 0.80$) in 2% (w/v) glucose/CMM that contained 100 μ M [^{3} H]nucleoside alone or with a 10 mM concentration of potential competitive substrate. The cultures were incubated (shaking) at 30°C for 45 min, at which point 200- μ l portions of each culture were layered over oil and subjected to centrifugation as described in Fig. III 1. Data are expressed as percentage of uridine uptake observed in the absence of competitive substrate and are mean values $\pm$ SD for three determinations. Abbreviations are: Ura, uracil; Ino, inosine; Cyd, cytidine; Ado, adenosine; dThd, thymidine; deazaUrd, 3-deazauridine; ψ rd, pseudouridine; 5F-dUrd, 5-fluoro-2'-deoxyuridine; 5F-Urd, 5-fluorouridine; dUrd, 2'-deoxyuridine; Urd, uridine; none, no competitor.

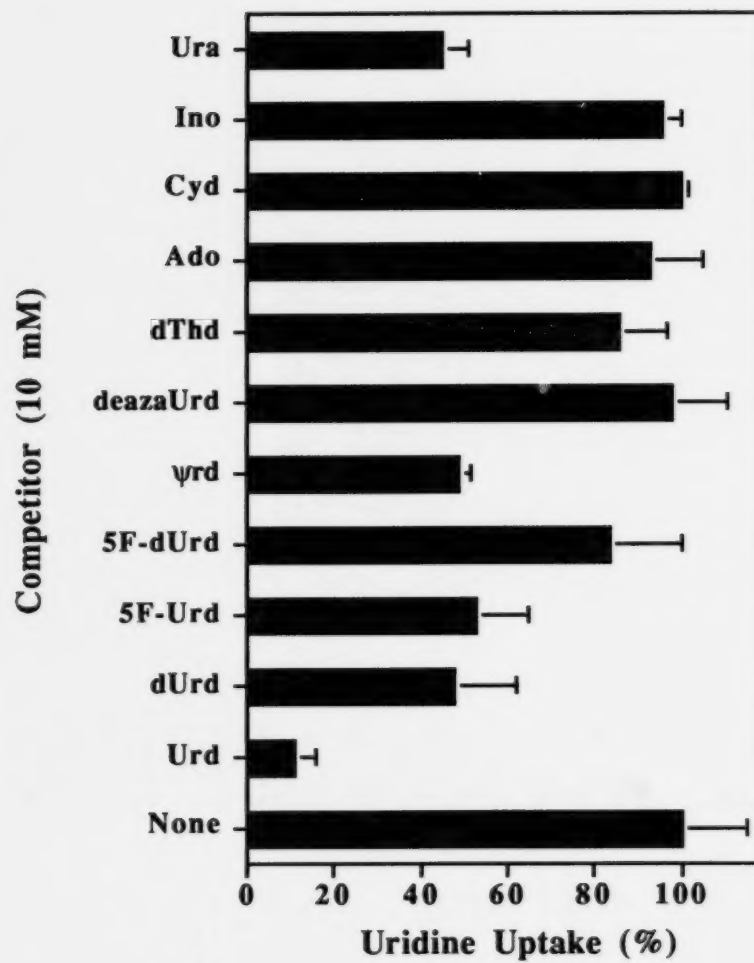


FIGURE III 3

H. simplex* Thymidine Kinase can be Functionally Expressed in Either a Constitutive or Inducible Manner in *S. cerevisiae

Yeast cells were examined for the presence of functional thymidine kinase in cell extracts using the *in vitro* assay described in Methods and Materials. Cell extracts were prepared from KY114 and KTK cells (KY114 cells transformed with the pDH01 vector) grown to identical OD₆₀₀ values in 2% galactose/CMM in the absence or presence of 100 μ M CuSO₄. The KY114 (■) and KTK (●) cell extracts were examined for the capacity to convert [³H]thymidine to [³H]thymidine-5'-monophosphate ([³H]dTMP) over a period of 0-90 min. The assay of thymidine kinase activity of KTK cell extracts was also conducted in the presence of a 250 μ M concentration of 2'-deoxyuridine (○), 2'-deoxycytidine (Δ), or thymidine-5'-triphosphate (dTTP; □). The activity of thymidine kinase ([³H]dTMP produced) in extracts prepared from cells grown in the absence (top panel) or presence (bottom panel) of CuSO₄ is presented as radioactivity per 12.5 μ l of cell extract. The data presented are individual assays from a single representative experiment.

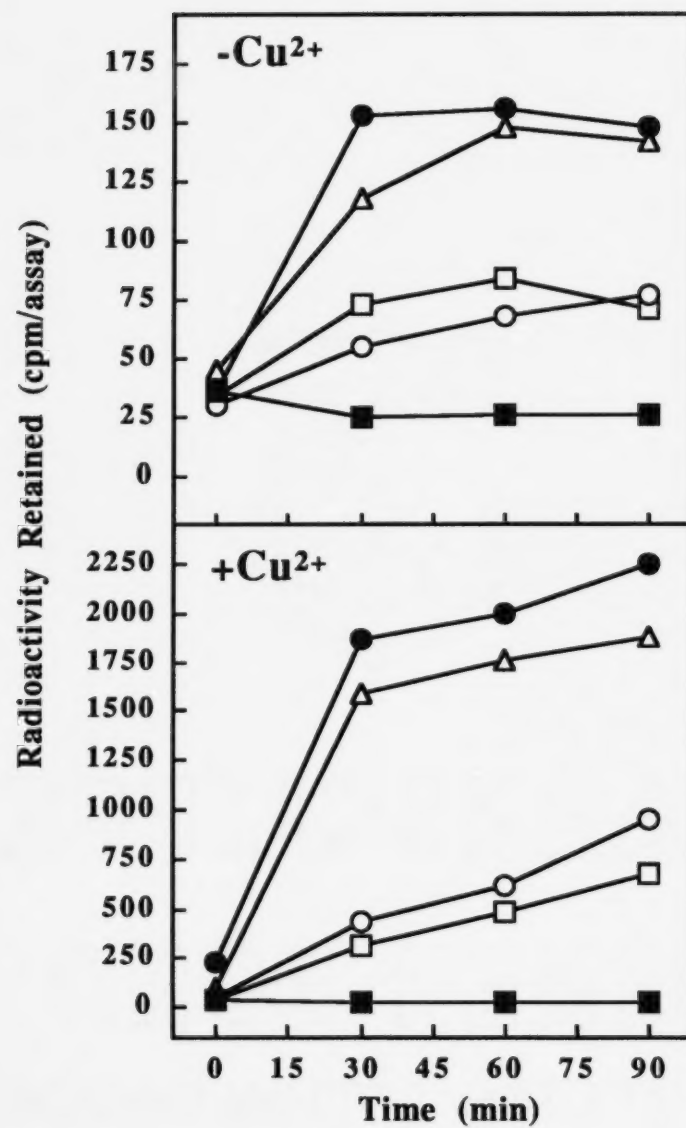


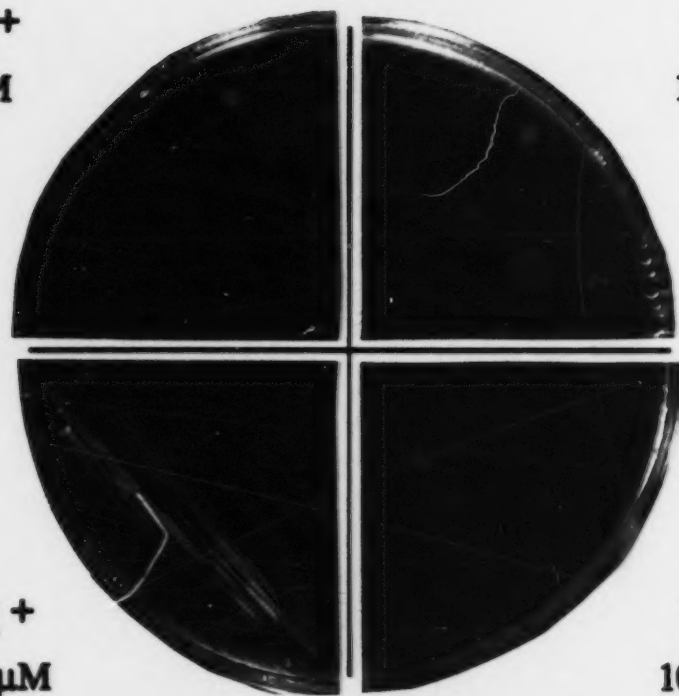
FIGURE III 4

***S. cerevisiae* That Express Thymidine Kinase Can be Rescued from dTMP Depletion by High Concentrations of Exogenous Thymidine**

A combination of intracellular thymidine kinase and high concentrations of exogenous thymidine can provide an alternate source of dTMP in yeast that lack functional thymidylate synthase. KY114 cells (TK⁻) and thymidine kinase-expressing KTK cells (TK⁺) were streaked onto galactose/CMM plates that contained methotrexate (50 µg/ml), sulfanilimide (6 mg/ml), and various concentrations of thymidine (0, 100, or 1000 µM). Plates were incubated at 30°C for 3-4 days.

TK +
0 μ M

TK +
100 μ M



TK +
1000 μ M

TK -
1000 μ M

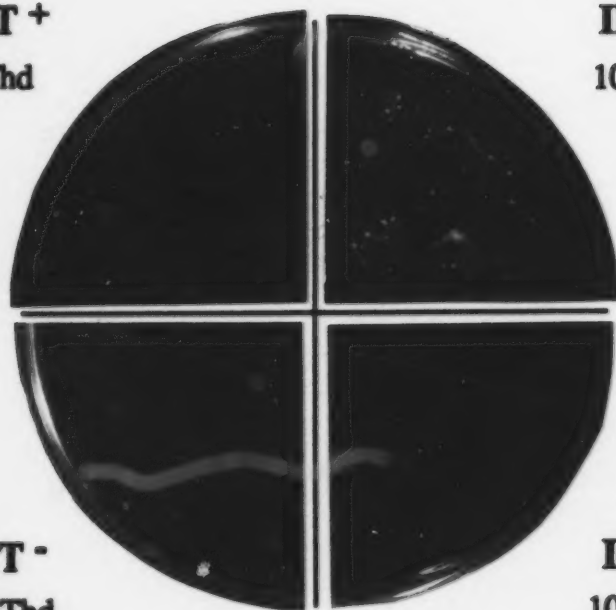
FIGURE III 5

Isolation of a cDNA Clone That Mediates Thymidine-Dependent Survival of TK-Expressing Yeast Under Conditions of Thymidylate Synthase Inhibition

A L1210/C2 cDNA library in the pYES2 yeast expression vector was transformed, in two separate experiments, into the KTK yeast strain and individual transformants were isolated using the expression cloning strategy described in the Results section. On the basis of restriction enzyme analysis, a single identical species of cDNA insert was isolated in both independent screening experiments. The KTK yeast strain was transformed with pYES2 containing (INSERT⁺) or lacking (INSERT⁻) the cDNA insert and plated onto 2% galactose/CMM plates that contained methotrexate (50 μ g/ml), sulfanilimide (6 mg/ml), and thymidine (0 or 100 μ M). The two transformants were also plated onto identical medium that contained 100 μ M concentration of thymidine-5'-monophosphate (dTMP) in place of thymidine. Plates were incubated at 30°C for 3-4 days.

INSERT +
0 μ M dThd

INSERT +
100 μ M dThd



INSERT -
100 μ M dThd

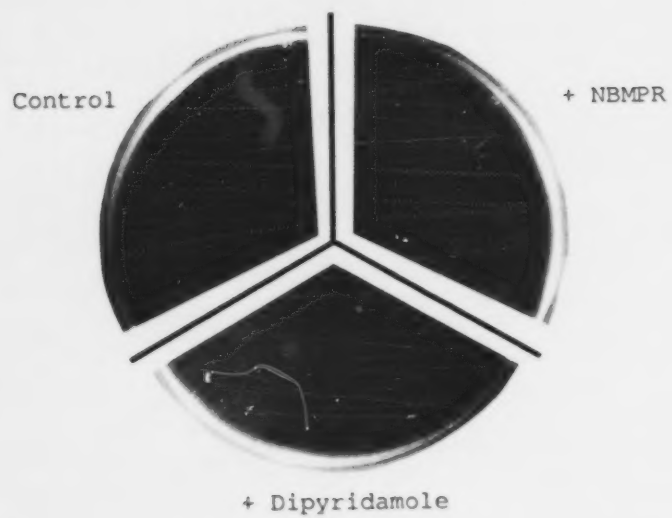
INSERT +
100 μ M dTMP

FIGURE III 6

Survival Mediated by the Mouse Transporter Protein 1 (*MTP1*) Clone is Not Inhibited by NBMPR and Dipyrindamole

KTK cells were transformed with the pMTP1 and pYES2 vectors and were plated onto 2% galactose/CMM plates that contained methotrexate (50 $\mu\text{g/ml}$), sulfanilimide (6 mg/ml), and 100 μM thymidine as well as 25 μM NBMPR, 10 μM dipyrindamole or no inhibitor (None). Plates were incubated at 30°C for 3 days.

pYES2



pMTP1

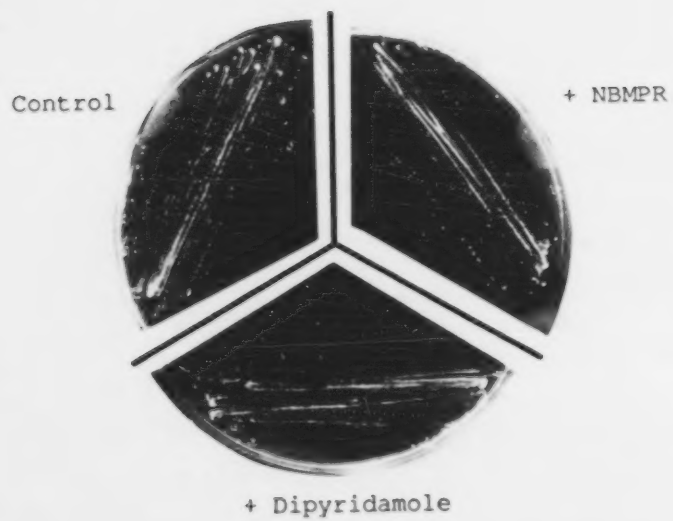


FIGURE III 7

Northern Blot Analyses for *MTP* mRNA Transcript

Total RNA was isolated from various sources and electrophoretically resolved on 1.2% agarose-0.67% formaldehyde gels, then transferred to Hybond-N membranes. The 960 bp HindIII-XbaI fragment of the *MTP1* cDNA was random primer-labeled with ^{32}P and hybridized to the RNA blots as described in Methods and Materials. The hybridized blots were washed twice (15 min each, RT) in 2X SSC/0.1% SDS and once (30 min, 37°C) in 0.2X SSC/0.1% SDS prior to being exposed to autoradiographic film.

Panel A. *MTP* mRNA is expressed in many cultured cell types. Equal portions (20 µg) of total RNA were subjected to Northern blot analysis after isolation from: mouse leukemia L1210/C2, L1210/B23, and L1210/MA27 cells, mouse lymphoma S49 and AE1 cells, human carcinoma HeLa cells, human leukemia HL60 cells, rat carcinosarcoma W256 cells, and *S. cerevisiae* (strain KY114) cells. The arrow indicates the migratory position of the mouse 18S rRNA (1870 nt).

Panel B. *MTP* mRNA is expressed at varying levels in different mouse tissues. Total RNA was isolated from the various tissues of a BALB/c mouse in the laboratory of C. Bleakley (University of Alberta) and equal portions (5 µg) were subjected to Northern blot analysis as described above. The arrow indicates the migratory position of the mouse 18S rRNA (1870 nt).

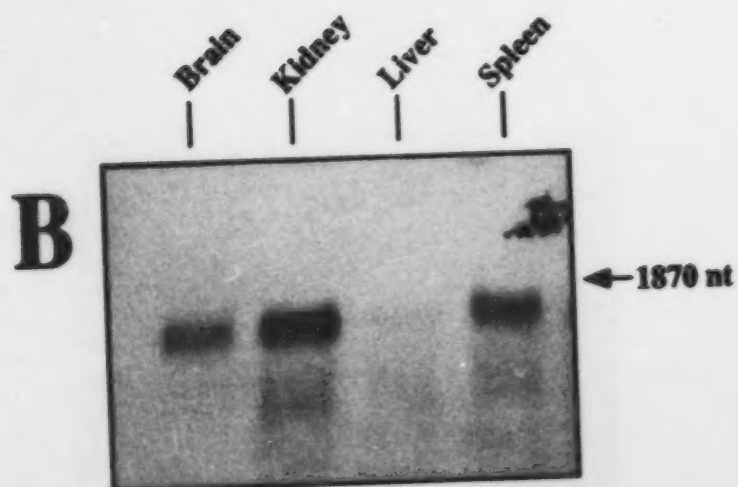
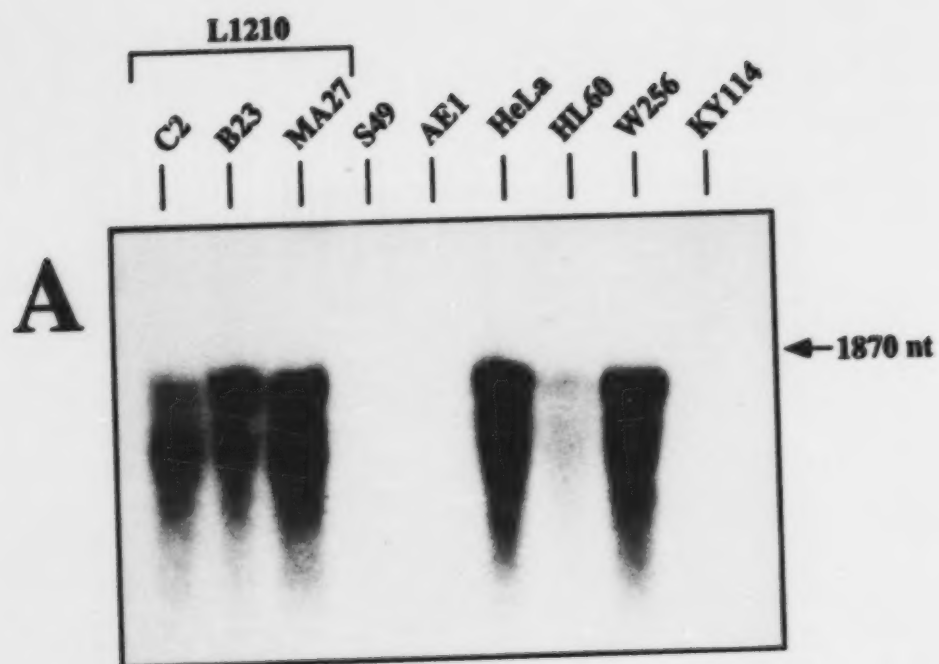


FIGURE III 8

Nucleotide Sequence and Predicted Amino Acid Sequence for the Open Reading Frame of the L1210/C2 Mouse Transporter Protein (*MTP*) cDNA

The 1435 nucleotide *MTP* cDNA sequence was derived from the sequences of pMTP1 (nucleotides 384-1435) and fragments cloned by anchor-PCR of the L1210/C2 cDNA library (nucleotides 35-1435) and a mouse brain λ GT11 cDNA library (nucleotides 1-34). The DNA sequencing reactions were performed on each strand using synthesized oligonucleotide primers to determine the cDNA sequence. Shown are the assigned translational start (ATG) and stop (TGA) codons of the predicted amino acid sequence for the longest open reading frame. A consensus polyadenylation signal sequence (AATAAA) is underlined and the ATTTA sequences, which act as mRNA destabilizing signals, are indicated in boxes. A potential N-linked glycosylation consensus sequence (Asn-X-Ser/Thr) is indicated by *. Consensus phosphorylation sequence motifs for cAMP kinase (cAMP-K) and casein kinase II (CaK II) are labeled and underlined, whereas the consensus sequence for protein kinase C (PKC) is labeled and indicated by shading.

[illegible]

FIGURE III 9

The Mouse Transporter Protein (*MTP*) cDNA Exhibits Four Hydrophobic Regions

The 219-amino acid protein predicted by the ORF of the *MTP3* cDNA shown in Fig. III 8 was examined for relative hydrophilicity by the method of Kyte and Doolittle (1982). The hydrophilic analysis employed an amino acid window of 11 residues and was performed using the Kyte-Doolittle algorithm of a MacVector Version 4.0 software program. Shown is the hydrophilic index as a function of amino acid residue. Hydrophobic regions are indicated by a negative hydrophilicity index.

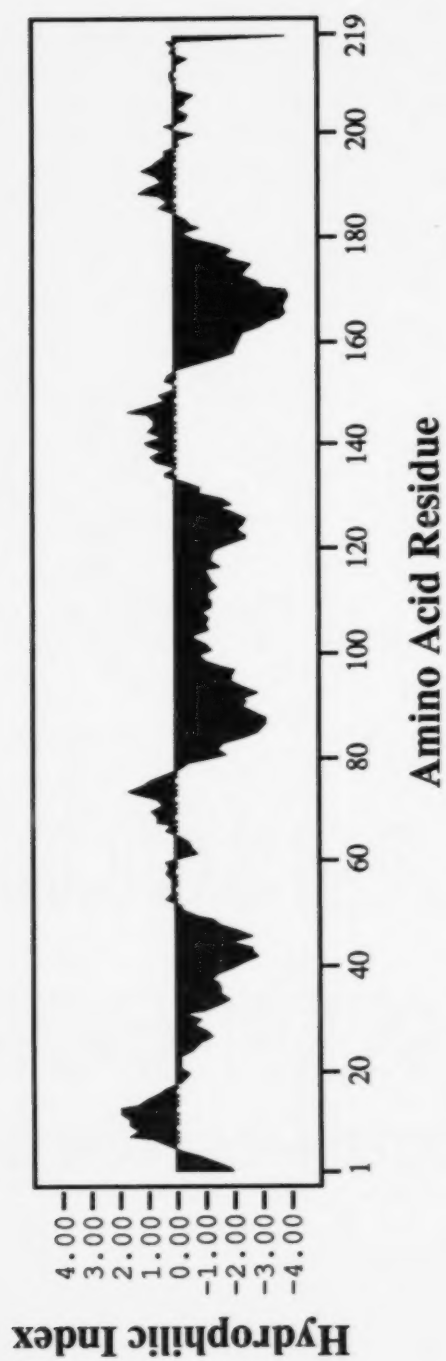


FIGURE III 10

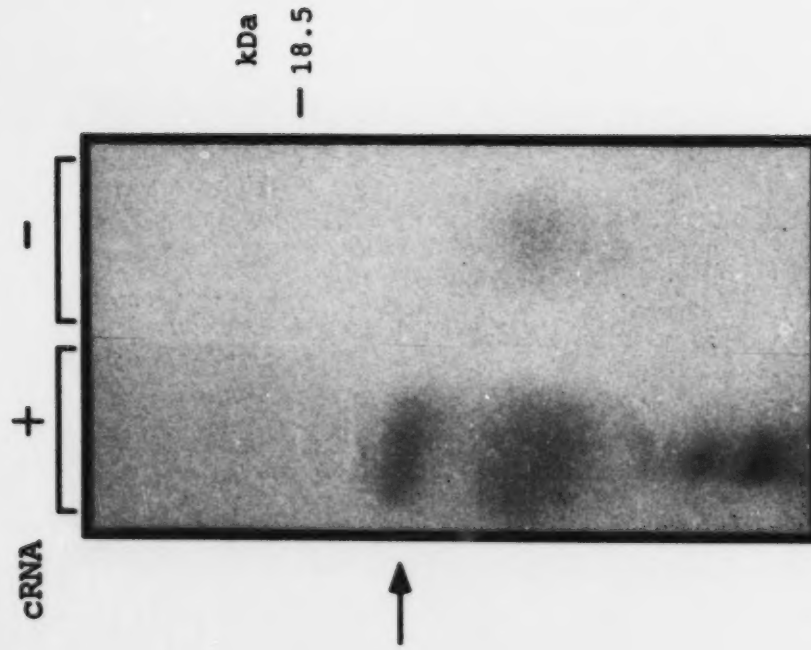
Identification of *MTP1* and *MTP3* cDNA-Encoded Proteins by *in vitro* Transcription and Translation

The proteins encoded by the *MTP1* and *MTP3* cDNAs were identified as *in vitro* transcription/translation products on SDS-polyacrylamide gels using the procedure detailed in Methods and Materials.

Panel A. The *MTP1* cDNA encodes a protein of near-predicted size. The pMTP1 vector was linearized at its unique NotI restriction site and utilized as DNA template in a T7 RNA polymerase-directed transcription reaction to generate complementary RNA (cRNA). Rabbit reticulocyte lysates containing ^{35}S -methionine were incubated in the presence (+) or absence (-) of 1 μg of *MTP1* cRNA. The translation reactions were subjected to SDS-PAGE on 12% polyacrylamide gels under reducing conditions and ^{35}S -labeled proteins were visualized by autoradiography. Prestained Low Range SDS-PAGE protein standards (lysozyme, 18.5 kDa; trypsin inhibitor, 27.5 kDa; carbonic anhydrase, 32.5; ovalbumin, 49.5 kDa; bovine serum albumin, 80 kDa; phosphorylase B, 106 kDa) were run in adjacent lanes. The migratory position of lysozyme (18.5 kDa) is indicated. The migratory position of the *in vitro* translated *MTP1*-encoded protein is indicated by the arrow and corresponded to approximately 16 kDa.

Panel B. A protein of 23.5 kDa is encoded by the *MTP3* cDNA. The pMTP3 vector was linearized at its unique NotI restriction site and used to generate *in vitro* translated ^{35}S -labeled proteins as described above. Translation reactions were performed in the presence (+) or absence (-) of 1 μg of *MTP3* cRNA. Prestained Low Range SDS-PAGE protein standards were run in adjacent lanes and their migratory positions are indicated. The migratory position of the *in vitro* translated *MTP3* protein is indicated by the arrow and corresponded to 23.5 kDa.

A. MTP1 CRNA



B. MTP3 CRNA

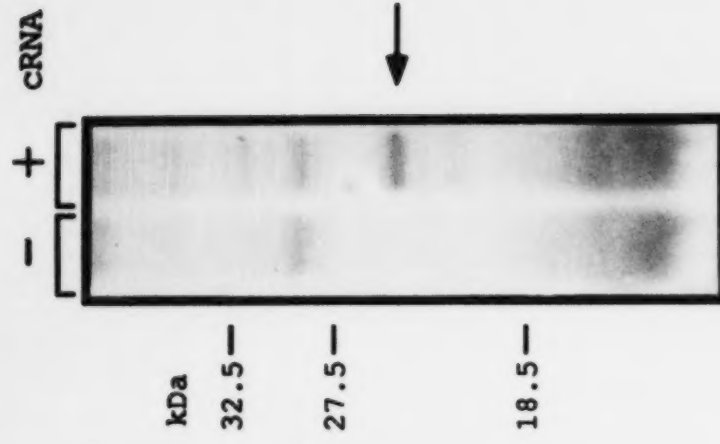


FIGURE III 11

The *HUMORF13* cDNA Encodes a Human Homolog of the Mouse Transporter Protein (*MTP*) cDNA

The nucleotide and predicted amino acid sequences of the ORF of the *MTP3* cDNA (bp 136-795 of Fig. III 8) were compared to nucleotide and protein databases of the National Center for Biotechnology Information using the BLAST network system. A single Genbank database entry, *HUMORF13* (Accession D14696), exhibited significant identity with the *MTP3* cDNA. Shown is an optimal alignment of the nucleotide sequences of the predicted ORFs performed using the BESTFIT program of the GCG program.

MTP	1	ATGGTGTCCATGACTTTCAAACGGAGCCGCGAGTGACCGGTTCTACAGCAC	50
HUMORF13	147	atggtgtccatgagtttcaagcgaaccgcagtgaccggttctacagcac	196
MTP	51	CCGGTGTGCGGCTGTTTCCATGTCCGCACCGGACGATCATCCTGGGGA	100
HUMORF13	197	cgggtgctgcggtgttgccatgtccgcaccgggacgatcatcctgggga	246
MTP	101	CCTGGTACATGGTGGTCAACCTGTTGATGGCCATTTTGCTGACCGTGGAG	150
HUMORF13	247	cctggtacatggtagtaaacctattgatggcaattttgctgactgtggaa	296
MTP	151	GTGACTCATCCAAATTCATGCCAGCTGTCAACATTCAGTATGAAGTCAT	200
HUMORF13	297	gtgactcatccaaactccatgccagctgtcaacattcagtatgaagtcac	346
MTP	201	TGGTAATTACTATTCTCTGAGAGAATGGCTGATAATGCCTGCGTTCTTT	250
HUMORF13	347	cggtaattactattcgtctgagagaatggctgataatgcctgtgttcttt	396
MTP	251	TTGCCGTCTCTGTTCTTATGTTTATAATCAGTTCAATGCTGGTTTATGGA	300
HUMORF13	397	ttgccgtctctgttcttatgtttataatcagttcaatgctggtttatgga	446
MTP	301	GCCATTTCTTATCAAGTGGGCTGGCTGATTCTTTCTTCTGCTACCGACT	350
HUMORF13	447	gcaatttcttatcaagtgggtggctgattccattcttctgttaccgact	496
MTP	351	CTTTGACTTCGTTCTCAGTTGTCTGGTGGCCATCAGTTCTCTCACTTACT	400
HUMORF13	497	ttttgacttcgtcctcagttgcctggttgctattagttctctcacctatt	546
MTP	401	TGCCAAGAATCAAAGAATATCTGGATCAGTTGCCTGATTTCCCATACAAA	450
HUMORF13	547	tgccaagaatcaaagaatatctggatcaactacctgattttccctacaaa	596
MTP	451	GATGACCTCCTGGCATTGGACTCCAGCTGCCTCCTGTTTCATCGTCCTTGT	500
HUMORF13	597	gatgacctcctggccttggaactccagctgcctcctgttcattgttctgt	646
MTP	501	GTTCTTTGTGGTATTCATCATTTTTAAGGCTTACCTAATCAATTGTGTTT	550
HUMORF13	647	gttctttgccttattcatcatttttaaggcttatctaattaactgtgttt	696
MTP	551	GGGAACCTGCTACAAATACATCAACAACAGAAATGTGCCAGAGATTGCCG	600
HUMORF13	697	gg..aactgctataaatacatcaacaaccgaaacgtgccggagattgctg	744
MTP	601	TGTACCCTGCCTTTGAAACACCTCCACAGTATGTTTGTGCCAACCTATGAA	650
HUMORF13	745	tgtaccctgcctttgaagcacctcctcagtacgttttgccaacctatgaa	794
MTP	651	ATGGCTGTGA	657
HUMORF13	795	atggccgtgaaaatgcctgaaaaagaaccaccacctccttacttacctgc	801
HUMORF13	802	ctga	805

FIGURE III 12

Nucleotide Sequence of the Putative Promoter Region of the Mouse Transporter Protein (*MTP*) Gene

A mouse spleen λ FIX II genomic DNA library was hybridization screened as described in Methods and Materials with a *MTP1* cDNA-derived probe and an oligonucleotide probe (5' CCGGTGCGGACATGGAAACAG 3') that corresponded to a region near the 5' terminus of the *MTP* cDNA coding region. A single phage clone containing a 20-kbp insert was isolated. A 703-bp HindIII insert fragment was identified and subjected to DNA sequencing after subcloning into the pBSSK⁺ vector. The HindIII fragment encompassed (i) 610 bp of promoter region and (ii) the first 89 nucleotides of the *MTP3* cDNA sequence. Shown is the sequence of the genomic DNA region (bp 1-610) in conjunction with the *MTP3* cDNA sequence (bp 611-771). The cDNA sequence is shown in lower case and the first 9 codons of its predicted ORF are indicated. The genomic DNA sequence was examined for the presence of transcription factor binding sequences (Faisst and Meyer, 1992) using the MacVector computer program. Regions of DNA sequence that encompass perfect consensus binding sites are labeled and their respective consensus sequences are indicated within shaded regions or by underlining. A putative CAAT sequence motif (CCAAT) is shown in bold font and a G/C box (GGGCGG) is enclosed in a box.

1 TACTCCACTTTATCATGCTGTCATTTTCCTTAATCCCTTGGGCTTTGGCA 50
 51 GAGTCCAGTGGTATTTATTGGCTCCCTCTAAGTAGGCTTCAGAGAGATGG 100
 101 GGAAGGACAGAAATCCAATCCTTTAGCCTACTTGCGAGCCCTCCTTTCT 150
 151 GGTCTGAGCCTAGAGGGCCACGCCAGAATGGTAGCACGCCCTTCCCACA 200
 201 GGACGCCCCGACAGGCCCAACAATAGAGCACGAATACGTACGTGGGCCT 250
 251 CGCACGCCCCGACAGGCCAACAATAGAGCACGAATACGTACGTGGGCCTC 300
 301 GCAGTGACAAAGACAACGTGGCGAAAGACAGCGCCAAAAATCTCCGTGCC 350
 351 CGCTGTCTGCCACCAACTCCGTCTTGTTCACCTTCTCCCTTGCGGAGC 400
 401 TCGTCTGGGAGACGGTGAATTACCGAGTTACCCTCAATTCCTACAGCCCC 450
 451 CGACAGCGAGCCCAAGCCACGCGCACCGCGGTCAAACAGCGCCGGAGAGAG 500
 501 TTGAACTTTGTATTGGGCGTCTGTATGTGGCAGAAGGCGGAGGGAGAGAT 550
 551 TCCGAGCCGACGTCCCGCGAGAGCCCGGGCCAGAGCGCAGGCCGACA 600
 601 GGAGTTGGCAagggcccgaggcggcgcagaagactccgtcctttg 650
 651 gctgtggacaccgtcgtagagtcgtctgggtgaaccgctgggcccgaag 700
 701 cttttgtcaggggcctgggcgagaacgagggccgcgaggttcgg atg 747
 748 gtg tcc atg act ttc aaa cgg agc 771

BIBLIOGRAPHY

- Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakalima, T., Hirano, T., and Kishimoto, T. (1990) A nuclear factor for IL6 expression (NF-IL6) is a member of the C/EBP family. *EMBO J.* **9**, 1897-1906.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410.
- Amara, S. G. (1992) A tale of two families. *Nature* **360**, 420-421.
- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1990) Current protocols in molecular biology. Greene Publishing and Wiley Interscience, New York.
- Baker, A. and Schatz, G. (1987) Sequences from a prokaryotic genome or mouse dihydrofolate reductase gene can restore the import of a truncated precursor protein into yeast mitochondria. *Proc. Natl. Acad. Sci. USA* **84**, 3117-3121.
- Barnard, E. A. (1992) Receptor classes and the transmitter-gated ion channels. *Trends Biochem. Sci.* **17**, 368-374.
- Becker, D.M. and Guarrente, L. (1989) High efficiency transformation of yeast by electroporation. *Methods in Enzymology* **194**, 182-187.
- Belt, J. A. (1983) Two types of transport activity in L1210 and other cultured neoplastic cells. *Mol. Pharm.* **24**, 479-484.
- Belt, J. A. and Noel, D. L. (1985) Nucleoside transport in Walker 256 rat carcinosarcoma and S49 mouse lymphoma cells. *Biochem. J.* **232**, 681-688.
- Bernstiel, M. L., Busslinger, M., and Strub, K. (1985) Transcription termination and 3' processing: the end is in site! *Cell* **41**, 349-359.
- Bibi, E. and Kaback, R. H. (1992) Functional complementation of internal deletion mutants in the lactose permease of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **89**, 1524-1528.
- Birnboim, H.C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl. Acids Res.* **7**, 1513-1523.
- Blake, M. C., Jambou, R. C., Swick, A. G., Kahn, J. W., and Azizkhan, J. C. (1990) Transcriptional initiation is controlled by upstream GC-box interactions in a TATAA-less promoter. *Mol. Cell. Biol.* **10**, 6632-6641.
- Boleti, B. (1991) Nucleoside transport in K562 leukemia cells. Ph.D. thesis, Department of Biochemistry, University of Alberta, Edmonton, Alberta.
- Cass, C. E., Kolassa, N., Uehara, Y., Dahling - Harley, E., Harley, E. R., and Paterson, A. R. P. (1981) Absence of binding sites for the transport inhibitor nitrobenzylthioinosine on nucleoside transporter-deficient mouse lymphoma cells. *Biochim. Biophys. Acta.* **649**, 769-777.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294-5299.

Chodosh, L. A., Baldwin, A. S., Carthew, R. W., and Sharp, P. A. (1988) Human CCAAT-binding proteins have heterologous subunits. *Cell* **53**, 11-24.

Cohen, A., Ullman, B., and Martin, D. W. Jr. (1979) Characterization of a mutant mouse lymphoma cell with deficient transport of purine and pyrimidine nucleosides. *J. Biol. Chem.* **254**, 112-116.

Crawford, C. R. and Belt, J. A. (1991) Sodium-dependent, concentrative nucleoside transport in Walker 256 rat carcinosarcoma cells. *Biochem. Biophys. Res. Commun.* **175**, 846-851.

Crawford, C. R., Ng, C. Y. C., and Belt, J. A. (1990a) Isolation and characterization of a L1210 cell line retaining the sodium-dependent carrier *cif* as its sole nucleoside transport activity. *J. Biol. Chem.* **265**, 13730-13734.

Crawford, C. R., Ng, C. Y. C., Noel, D., and Belt, J. A. (1990b) Nucleoside transport in L1210 murine leukemia cells. *J. Biol. Chem.* **265**, 9732-9736.

Dagnino, L (1988) Sodium-driven nucleoside transport in mouse leukemia L1210 cells. Ph.D. thesis, Department of Pharmacology, University of Alberta, Edmonton, Alberta.

Dagnino, L (1988) Sodium-driven nucleoside transport in mouse leukemia L1210 cells. Ph.D. thesis, Department of Pharmacology, University of Alberta, Edmonton, Alberta.

Davis, L.G., Dibner, M.D., and Battey, J.F. (1986) Basic methods in molecular biology. Elsevier Science Publishing Co.

Elble, R. (1992) A simple and efficient procedure for transformation of yeasts. *BioTechniques* **13**, 18-20.

Ellison, M. J. and Hochstrasser, M. (1991) Epitope-tagged ubiquitin. *J. Biol. Chem.* **266**, 21150-21157.

Faisst, S. and Meyer, S. (1992) Compilation of vertebrate-encoded transcription factors. *Nucleic Acids Res.* **20**, 3-26.

Favre, D. (1992) Improved phenol-based method for the isolation of DNA fragments from low melting temperature agarose gels. *BioTechniques* **13**, 22-26.

Foury, F. and Goffeau, A. (1975) Stimulation of active uptake of nucleosides and amino acids by cyclic adenosine 3':5'-monophosphate in the yeast *Schizosaccharomyces cerevisiae*. *J. Biol. Chem.* **250**, 2354-2362.

Frommer, W. B., Hummel, S., and Reismeier, J. W. (1993) Expression cloning in yeast of a cDNA encoding a broad specificity amino acid permease from *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **90**, 5944-5948.

Fujita, N., Sweet, M. T., Fox, T. D., Nelson, N., Claudio, T., Linstrom, J. M., and Hess, G. P. (1986) Expression of cDNAs for acetylcholine receptor subunits in the yeast cell plasma membrane. *Biochem. Soc. Symp.* **52**, 41-56.

- Gardiner-Garden, M. and Frommer, M. (1987) CpG islands in vertebrate genomes. *J. Mol. Biol.* **196**, 261-282.
- Gati, W. P., Misra, H. K., Knaus, E. E., and Wibe, L. I. (1984) Structural modifications at the 2'- and 3'-positions of some pyrimidine nucleosides as determinants of their interaction with the mouse erythrocyte nucleoside transporter. *Biochem. Pharmacol.* **33**, 3325-3331.
- Ghosh, P. K., Lebowitz, P., Frisque, F. J., and Gluzman, Y. (1981) Identification of a promoter component in positioning the 5' termini of simian virus SV40 early mRNAs. *Proc. Natl. Acad. Sci. USA*, **78**, 100-104.
- Grenson, M. (1969) The utilization of exogenous pyrimidines and the recycling of uridine-5'-phosphate derivatives in *Saccharomyces cerevisiae*, as studied by means of mutants affected in pyrimidine uptake and metabolism. *Eur. J. Biochem.* **11**, 249-260.
- Griffith, J. K., Baker, M. E., Rouch, D. A., Page, M. G. P., Skurray, R. A., Paulsen, I. T., Chater, K. F., Baldwin, S. A., and Henderson, P. J. F. (1992) Membrane transport proteins: implications of sequence comparisons. *Curr. Opin. Cell Biol.* **4**, 684-695.
- Grivell, A. R. and Jackson, J. F. (1968) Thymidine kinase: Evidence for its absence from *Neurospora crassa* and some other micro-organisms and the relevance of this to the specific labeling of deoxyribonucleic acid. *J. Gen. Microbiol.* **54**, 307-317.
- Gunekunst, K. A., Rao, G. N., and Church, R. L. (1990) Molecular cloning and complete nucleotide sequence of the cDNA encoding a bovine lens intrinsic membrane protein (MP19). *Curr. Eye Res.* **9**, 955-961.
- Hart, G. W. (1992) Glycosylation. *Curr. Opin. Cell Biol.* **4**, 1017-1023.
- Hebert, D. N. and Carruthers, A. (1992) Uniporters and anion antiporters. *Curr. Opin. Cell Biol.* **3**, 702-709.
- Hogue, D. L. and Cass, C. E. (1994) *Spodoptera frugiperda* (Sf9) cells express novel purine- and pyrimidine-selective nucleoside transporters. *Insect Biochem. Mol. Biol.* In Press.
- Hogue, D. L., Hodgson, K. C., and Cass, C. E. (1990) Effects of inhibition of N-linked glycosylation by tunicamycin on nucleoside transport polypeptides of L1210 leukemia cells. *Biochem. Cell Biol.* **68**, 199-209.
- Hsu, L. C., Chiou, T. J., Chen, L., and Bush, D. R. (1993) Cloning a plant amino acid transporter by functional complementation of a yeast amino acid transport mutant. *Proc. Natl. Acad. Sci. USA* **90**, 7441-7445.
- Jones, E. W. and Fink G. R. (1982) Regulation of amino acid and nucleotide biosynthesis in yeast. In: The molecular biology of the yeast *Saccharomyces*, metabolism and gene expression. pp. 181-299. Cold Spring Harbor Laboratory
- Jones, K. A., Kadonaga, J. T., Rosenfeld, P. J., Kelly, T. J., and Tijan, R. (1987) A cellular DNA-binding protein that activates eucaryotic transcription and DNA replication. *Cell* **48**, 79-89.

Kadonaga, J. T., Jones, K. A., and Tijan, R. (1986) Promoter-specific activation of RNA polymerase II transcription by Sp1. *Trends Biochem. Sci.* **11**, 20-23.

Kaldi, K., Diestelkotter, P., Stenbeck, G., Auerbach, S., Jakle, U., Magert, H. J., Wieland, F. T., and Just, W. W. (1993) Membrane topology of the 22 kDa integral peroxisomal membrane protein. *Fed. Eur. Biochem. Soc.* **315**, 217-222.

Kenelly, P. J. and Krebs, E. G. (1991) Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem.* **266**, 15555-15558.

Kozak, M. (1991) An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell Biol.* **115**, 887-903.

Kuchler, K. and Thorner, J. (1992) Functional expression of human mdrl in the yeast *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **89**, 2302-2306.

Kumar, N. M., Jarvis, L. J., Tenbroek, E., and Louis, C. F. (1993) Cloning and expression of a major rat lens membrane protein, MP20. *Exp. Eye Res.* **56**, 35-43.

Kwong, F. Y. P., Davies, A., Tse, M. C., Young, J. D., Henderson, P. J. F., and Baldwin, S. A. (1988) Purification of the human erythrocyte nucleoside transporter by immunoaffinity chromatography. *Biochem. J.* **255**, 243-249.

Kyte, J. and Doolittle, R. F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105-132.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of bacteriophage T4. *Nature* **227**, 680-685.

Lemmens, M., Verheyden, K., Van Veldhoven, P. P., Vereecke, J., Mannaerts, G. P., and Carmeliet, E. (1989) Single-channel analysis of a large conductance channel in peroxisomes from rat liver. *Biochim. Biophys. Acta.* **984**, 351-359.

Little, J. G. and Haynes, R. H. (1979) DNA-specific labeling in yeast mutants. *Genetics*, **74**, S161.

Lo, K., Landau, N. R., and Smale, S. T. (1991) LyF-1, a transcriptional regulator that interacts with a novel class of promoters for lymphocyte-specific genes. *Mol. Cell. Biol.* **11**, 5229-5243.

Lu, C., Yang, Y. F., Ohashi, H., and Walfish, P. G. (1990) *In vivo* expression of rat liver c-erbA β -thyroid hormone receptor in yeast (*Saccharomyces cerevisiae*). *Biochem. Biophys. Res. Commun.* **171**, 138-142.

Mak, P., McDonnell, D. P., Weigel, D. P., Schrader, W. T., and O'Malley, B. W. (1989) Expression of functional chicken oviduct progesterone receptors in yeast (*Saccharomyces cerevisiae*). *J. Biol. Chem.* **264**, 21613-21618.

Marger, M. D. and Saier, M. H. Jr. (1993) A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. *Trends Biochem. Sci.* **18**, 13-20.

- Mattila, P., Korpela, J., Tenkanen, T., and Pitkanen, K. (1991) Fidelity of DNA synthesis by the *Thermococcus litoralis* DNA polymerase - an extremely heat stable enzyme with proofreading activity. *Nucleic Acids Res.* **19**, 4967-4973.
- McKenna, E., Hardy, D., and Kaback, R. H. (1992) Insertional mutagenesis of hydrophilic domains in the lactose permease of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **89**, 11954-11958.
- McNeil, J.B. and Friesen, J.D. (1981) Expression of the *Herpes simplex* virus thymidine kinase gene in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **184**, 386-393.
- Means, A. L. and Farnham, P. J. (1990) Transcription initiation from the dihydrofolate reductase promoter is positioned by HIP1 binding at the initiation site. *Mol. Cell. Biol.* **10**, 653 - 661.
- Momura, N., Miyajima, N., Kawarabayashi, Y., and Tabata, S. (1993) Prediction of new human genes by entire sequencing of randomly sampled cDNA clones. Unpublished Kazusa DNA Research Institute, Japan.
- Morimyo, M., Hongo, E., Hama-Inaba, H., and Machida, I. (1992) Cloning and characterization of the *mvrC* gene of *Escherichia coli* K-12 which confers resistance against methyl viologen toxicity. *Nuc. Acids Res.* **20**, 3159-3165.
- O'Donovan, G. A. (1978) Thymidine metabolism in bacteria (and how or how not to label DNA). In: *DNA Synthesis: Present and Future*. (Molineux, I. and Kohiyama, M. eds.) Plenum, New York.
- Purewal, A. S. (1991) Nucleotide sequence of the ethidium efflux gene from *Escherichia coli*. *Fed. Eur. Microbiological. Soc. Microbiol. Letters* **82**, 229-232.
- Pearson, W. R. and Lipman, D. J. (1988) Improved tools for biological sequence analysis. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2444 - 2448.
- Raymond, M. Gros, P., Whiteway, M., and Thomas, D. Y. (1992) Functional complementation of yeast *ste6* by a mammalian multidrug resistance *mdr* gene. *Science*, **256**, 232-234.
- Reiesmeier, J. W., Willmitzer, L., and Frommer, W. B. (1992) Isolation and characterization of a sucrose carrier cDNA from spinach by functional expression in yeast. *EMBO J.* **11**, 4705-4713.
- Reizer, J., Reizer, A., and Saier, M. H. Jr. (1993) The MIP family of integral membrane channel proteins: sequence comparisons, evolutionary relationships, reconstructed pathway of evolution, and proposed functional differentiation of the two repeated halves of the proteins. *Crit. Rev. Biochem. Mol. Biol.* **28**, 235-257.
- Riggs, K. J., Merrell, K. T., Wilson, G., and Calame, K. (1991) Common factor 1 is a transcriptional activator which binds in the *c-myc* promoter, the skeletal α -actin promoter, and the immunoglobulin heavy-chain enhancer. *Mol. Cell. Biol.* **11**, 1765-1769.
- Romanos, M. A., Scorer, C. A., and Clare, J. J. (1992) Foreign gene expression in yeast: a review. *Yeast* **8**, 423-488.

- Runswick, M. J., Walker, J. E., Bisaccia, F., Iacobazzi, V., and Palmier, F. (1990) Sequence of the bovine 2-oxoglutarate/malate carrier protein: structural relationship to other mitochondrial transport proteins. *Biochem.* **29**, 11033-11040.
- Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1987) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487-491.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular cloning - a laboratory manual*. Cold Spring Harbour Laboratory.
- Sanger, F., Niksen, S., and Coulson, A.R. (1977) DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463 - 5467.
- Sasatsu, M., Shibata, Y., Noguchi, N., and Kono, M. (1992) High-level resistance to ethidium bromide and antiseptics in *Staphylococcus aureus*. *Fed. Eur. Microbiological. Soc. Microbiol. Letters* **93**, 109-114.
- Seed, B. and Aruffo, A. (1987) Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. *Proc. Natl. Acad. Sci. USA* **84**, 3365-3369.
- Sentenac, H., Booneaud, N., Minet, M., Lacroute, F., Salmon, J. M., Gaymard, F., and Grignon, C. (1992) Cloning and expression in yeast of a plant potassium ion transport system. *Science*, **256**, 663-665.
- Shaw, G. and Kamen, R. (1986) A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* **46**, 659-667.
- Singer, S. J. (1990) The structure and insertion of integral proteins in membranes. *Ann. Rev. Cell Biol.* **6**, 247-296.
- Sone, T., McDonnell, D. P., O'Malley, B. W., and Pike, J. W. (1990) Expression of human vitamin D receptor in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **265**, 21997-22003.
- Tabor, S. (1990) Expression using the T7 polymerase/promoter system. In: *Current Protocols in Molecular Biology* (Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds.). Greene Publishing and Wiley Interscience, New York.
- Van Veldhoven, P. P., Just, W. W., and Mannaerts, G. P. (1987) Permeability of the peroxisomal membrane to cofactors of β -oxidation. *J. Biol. Chem.* **262**, 4310-4318.
- Vijayalakshmi, D., Dagnino, L., Belt, J. A., Gati, W. P., Cass, C. E., and Paterson, A.R. P. (1992) L1210/B23.1 cells express equilibrative, inhibitor-sensitive nucleoside transport activity and lack two parental nucleoside transport activities. *J. Biol. Chem.* **267**, 16951-16956.
- Vijayalakshmi, D., Robbins, M. J., Belt, J. A., Gati, W. P., Cass, C. E., and Paterson, A.R. P. (1994a) Interaction of ribofuranosyl-modified analogs of adenosine with the NBMPR-sensitive nucleoside transporter. *In Preparation*.

Vijayalakshmi, D., Robbins, M. J., Belt, J. A., Gati, W. P., Cass, C. E., and Paterson, A.R. P. (1994b) Structural determinants of adenosine permeation via the NBMPR-insensitive nucleoside transporter. *In Preparation*.

Wasylyk, C., Flores, P., Gutman, A., and Wasylyk, B. (1989) PEA3 is a nuclear target for transcription activation by non-nuclear oncogenes. *EMBO J.* **8**, 3371-3378.

Weiher, H., Noda, T., Gray, D. A., Sharpe, A. H., and Jaenisch, R. (1990) Transgenic mouse model of kidney disease: insertional inactivation of ubiquitously expressed gene leads to nephrotic syndrome. *Cell* **62**, 425-434.

Zhu, X. L., Ward, C., and Wiessbach, A. (1984) Control of *Herpes simplex* virus thymidine kinase gene expression in *Saccharomyces cerevisiae* by a yeast promoter sequence. *Mol. Gen. Genet.* **194**, 31-41.

Chapter IV

FUNCTIONAL AND STRUCTURAL STUDIES OF *MTP*, A PUTATIVE GOLGI SUBSTRATE TRANSPORTER¹

¹ A version of this chapter has been submitted for publication: Hogue, D. L. , Ellison, M. J., Young, J. D. and Cass, C. E. (1994).

INTRODUCTION

The mouse transporter protein (*MTP*) was identified by its ability to functionally complement in a NT protein cloning method using *S. cerevisiae*. This yeast relies on the thymidylate synthase-mediated methylation of dUMP to obtain dTMP and lacks (i) a thymidine kinase and (ii) a mechanism for thymidine influx. The expression cloning method involved the expression of a mouse L1210/C2 cDNA library in thymidine kinase-expressing yeast under the conditions of (i) inhibition of *de novo* thymidylate synthase-mediated synthesis of dTMP and (ii) in the presence of thymidine. Under these conditions, yeast cells that expressed a cDNA-encoded protein which mediated thymidine influx would be expected to obtain exogenous thymidine and generate dTMP via the action of thymidine kinase. These yeast cells would therefore survive the normally lethal conditions of inhibition of *de novo* dTMP synthesis inhibition.

MTP is a 25.2-kDa protein that contains 4 potential membrane-spanning domains and does not exhibit homology with known substrate transporters. However, the hydrophobic topography of *MTP* resembles that of a number of other membrane proteins of eucaryotic cells including type I transmitter-gated ion channels (Barnard, 1992), the connexins or tight junction channels (Bennett *et al.*, 1991), the Mpv 17 protein (Weiher *et al.*, 1990), the peroxisomal membrane protein 22 (PMP 22) (Kaldi *et al.*, 1993) and the lens MP20 protein (Kumar *et al.*, 1993; Gutskunst *et al.*, 1990), as well as several bacterial transporters responsible for the cellular efflux of toxic compounds (Purewal, 1991; Morimyo *et al.*, 1992; Sasatsu *et al.*, 1992). The functions of the Mpv 17, MP20 and PMP22 proteins have not yet been directly demonstrated, although they have been suggested to affect the general permeability of membranes.

Work was undertaken to determine if *MTP* actually functions as a transporter and where it is expressed in the cell. This chapter describes (i) the generation of polyclonal antibodies specific for *MTP* and their use as immunological probes to identify *MTP* and its cellular location and (ii) the functional expression of *MTP* in *Xenopus laevis* oocytes to assess the capacity of this protein to mediate nucleoside uptake. Using this approach, *MTP* has been demonstrated to (i) function as a substrate transporter and, (ii) be localized in the Golgi compartment.

METHODS AND MATERIALS

Materials

Cell culture media (Fisher's, RPMI 1640, DMEM, MEM), calf serum, horse serum, fetal bovine serum, and gentamycin were from Collaborative Biomedical.; L-[4,5-³H]leucine, [¹⁴C(U)]sucrose, and [³H]H₂O were from DuPont Canada Inc.; D-[5,6-³H]uridine, D-[methyl-³H]thymidine, D-[3,8-³H]adenosine, and EcoLite scintillant were from ICN Biomedicals Inc.; dilazep hydrochloride was a gift from F. Hoffman-LaRoche; prestained protein molecular weight standards were from BioRad (Low Range SDS-PAGE Standards), Gibco/BRL (Prestained 6-60 kDa Protein Ladder) and Sigma (Dalton Mark VII Protein Standards); restriction enzymes, DNA modification enzymes and placental RNase inhibitor were from Gibco/BRL or New England Biolabs; m⁷G(5')ppp(5')G cap analog and *in vitro* transcription kits (T7 MessageMachine) were from Ambion; Bacto-Yeast Extract, Bacto-Casamino acids, Bacto-Peptone, Difco Bacto-Agar, and Bacto-Yeast Nitrogen Base were from Difco Inc.; bicinchoninic acid protein assay reagents A and B, sulfanilamide, and methotrexate were from Sigma Chemicals; Freund's complete and incomplete adjuvants were from Gibco/BRL; goat anti-rabbit IgG (H+L)-horseradish peroxidase (HRP) was from BioRad; donkey anti-rabbit IgG (H+L)-

rhodamine B was from Jackson Laboratories Inc.; CNBr-activated sepharose-CL was from Pharmacia; the pMalcRI vector was from New England Biolabs; the pcDNA1-AMP and pcDNA1-AMP-CAT vectors were from Invitrogen Corp.; the pCMV β Gal vector was from CloneTech; and other materials were obtained from standard commercial sources.

Mammalian Cell lines

Monkey kidney Cos-1 cells (ATCC) were grown by D. Mowles as adherent cultures in DMEM medium supplemented with NaHCO₃ (2 g/l) and 5% calf serum and 5% NuSerum IV. The origin and maintenance of other cell lines are described in Methods and Materials of Chapter III. All stock cultures were maintained in media without antibiotics at 37°C in 95% air and 5% CO₂ with 95% humidity. Cell concentrations of cultures were determined using a Coulter Counter Model ZF electronic particle counter (Coulter Electronics Inc.). Stock suspension cultures were diluted to 0.1 x 10⁵ - 0.2 x 10⁵ cells/ml every 2-3 days, and adherent cultures were subcultured to 0.1 x 10⁵ - 0.2 x 10⁵ cells/ml every 4-6 days.

***Saccharomyces cerevisiae* and *Escherichia coli* Strains**

The *S. cerevisiae* strain "KTK", the yeast growth medium employed in this study and the transformation of yeast with plasmid DNA are described in Methods and Materials of Chapter III.

The TOP10F' *Escherichia coli* strain (F'(tet^r), *mcrA*, Δ (*mmr-hsdRMS-mcrBC*) *p80dlacM15*, Δ *lacX74*, *deoR*, *recA1*, *araD139*, Δ (*ara,leu*)7697, *galU*, λ^- , *rpsL*, *endA1*, *nupG*) was used for plasmid propagation. Over-expression of *trpE*- and *malE*-fusion proteins was carried out using strain UT580 (Δ [*lac-pro*], *rK*⁻, *mK*⁺, *Tn10*, *supD*, *LacIq*, F', *traD*, *pro*⁺, *lacZ* Δ M15). The *E. coli* strains were grown in LB medium, as described in Methods and Materials of Chapter III.

***Xenopus laevis* Oocytes**

Mature stage VI oocytes were isolated from *X. laevis* in the laboratory of J. Young (University of Alberta) using established procedures (Huang *et al.*, 1993). Briefly, ovarian lobes were removed, opened, and washed into modified Barth's buffer (MBM; 88 mM NaCl, 1 mM KCl, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 2.4 mM NaHCO₃, 2.5 mM sodium pyruvate, 0.05 mg/ml penicillin, 0.1 mg/ml gentamycin sulfate, 10 mM HEPES pH 7.5). Oocytes were released from ovarian tissue by incubation (RT, approximately 2 hr) in MBM buffer containing collagenase (8 mg/ml, Type I) and trypsin inhibitor (1 mg/ml, Type III). Stage VI oocytes were selected and incubated (RT, 1 hr) in phosphate buffer (0.1% w/v bovine serum albumin (BSA), 100 mM K₂HPO₄ pH 6.5) to remove residual follicular layers. Healthy oocytes were maintained in MBM buffer at 18°C for approximately 24 hr, then used for microinjection (Inject+Matic microinjector). Oocytes were injected with either 20 nl sterile dH₂O (control) or 20 nl (10 ng) of RNA transcript in sterile dH₂O (0.5 ng/nl) and incubated in MBM at 18°C for 2-3 days, with daily changes of buffer, prior to use. Oocytes were harvested and injected by personnel in Dr. J. D. Young's laboratory (Department of Physiology, University of Alberta).

Permeant Uptake Studies in *X. laevis* Oocytes

Non-radioactive and radiolabeled permeants were prepared as described in Methods and Materials ("Permeant Uptake Studies in *S. cerevisiae*") of Chapter III. Stage VI oocytes isolated from *X. laevis* as described above were used in radiolabeled permeant uptake assays according to the method of Huang *et al.* (1993). Oocytes were distributed into the wells (10 oocytes per well) of a 24-well (flat bottom) tissue culture plate. The

bath buffer was aspirated and the period of uptake was initiated by adding to each well a 0.35-ml volume of transport buffer (100 mM NaCl, 20 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES pH 7.5) that contained radiolabeled permeant. The oocyte suspension was incubated with gentle shaking at RT during the period of uptake and was transferred to a 1.5 ml-microcentrifuge tube just prior to the end of the uptake period. The uptake interval was terminated by aspirating the radioactive medium, adding a 1 ml-portion of ice-cold transport buffer, aspirating the buffer and again repeating the wash step whereupon oocytes were suspended in a small volume of ice-cold transport buffer and individually placed into scintillation vials. The aspiration and washing steps were completed within 1 min. Oocytes were dissolved in 0.5 ml of 5% SDS by shaking at 37°C for 30 min. EcoLite scintillant (5 ml) was added to each vial and radioactive content was measured. Uptake values were calculated as means $\pm$ SD of 6-10 measurements, each conducted with a single oocyte.

Transient Transfection of Mammalian Cells

Expression of cDNA-encoded proteins in adherent cultures of mammalian cells was accomplished using the transient transfection procedure described by Ausubel *et al.* (1989). Actively proliferating cells were plated and grown to an estimated 50% confluency in 60-mm dishes, washed once with sterile PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄ pH 7.4), then 2 ml of fresh DMEM medium that contained 10% NuSerum and 100 μ M chloroquine was added to each dish. A 0.1-ml portion of DNA/dextran mixture (1 μ g plasmid DNA, 4 mg/ml DEAE-dextran, 137 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1.4 mM MgCl₂, 1.4 mM Na₂HPO₄, 25 mM Tris-HCl pH 7.5) was added dropwise, with swirling, into the dishes. The dishes were incubated at 37°C for 4 hr and then treated with dimethylsulfoxide (DMSO) as follows. The medium was removed and cells were incubated in a 2-ml portion of 10% (v/v) DMSO in PBS for 2 min at RT. The DMSO-containing PBS was removed and the cells were washed three times with PBS prior to the addition of fresh growth medium. The transfected cells were grown for a 24-hr period then trypsinized and plated at lower cell densities. Studies utilizing transfected cells were conducted 22-72 hr after transfection.

Efficiency of the transient transfection method was estimated using a β -galactosidase staining procedure (Lin and Culp, 1991) in which cells were transfected with the pcDNA1-AMP- β Gal vector. Adherent cells that had been transfected with 1-3 μ g of plasmid DNA were fixed by incubation (15 min, RT) in 0.5% glutaraldehyde in PBS and then washed three times with PBS. The cells were then incubated (16-36 hr, 37°C) in a staining mixture that contained 1 mg/ml 5-bromo-4-chloro-3-indoyl- β -D-galacopyranoside (X-Gal), 20 mM potassium ferricyanide, 20 mM potassium ferrocyanide, and 2 mM MgCl₂ in PBS. Transfected cells expressing β -galactosidase stained an aqua-blue color and the percentage of transfected cells in a particular culture was estimated by counting stained and unstained cells in several microscopic fields. Transfection efficiency varied between experiments (range 15-30%) and normally was 20%.

Isolation of Cellular Fractions From Yeast, Mammalian Cells and *Xenopus* Oocytes

Nucleus-free ("post-nuclear") cell fractions were prepared from adherent cultures of mammalian cells in a manner similar to that described by Ausubel *et al.* (1989). Briefly, the cultures (60-mm dishes, 4-5 dishes per preparation) were washed once with ice-cold PBS and cells were collected, by scraping with a plastic policeman, into a 1.5-ml volume of ice-cold lysis buffer (0.25 M sucrose, 1 mM PMSF, 10 mM HEPES pH 7.5). Cells were lysed by Dounce homogenization (50 strokes, 4°C) and centrifuged (1000 x g, 10 min, 4°C) to pellet the unbroken cells and nuclei. The supernatant was collected and then

subjected to centrifugation (30000 x g, 15 min, 4°C) to isolate the post-nuclear membrane (pellet) and soluble (supernatant) fractions.

Subcellular membrane fractions prepared from rat liver cells were obtained from J. Vance (University of Alberta) and included total cellular protein, enriched preparations of Golgi and (smooth and rough) endoplasmic reticulum, crude and purified preparations of mitochondria and mitochondrial-associated membranes. The smooth and rough endoplasmic reticulum and Golgi fractions were isolated by the method of Croze and Morre (1984), as modified by Vance and Vance (1988). Mitochondrial-associated membranes and the crude and pure fractions of mitochondria were prepared by the method of Vance (1990). The mitochondrial-associated membrane fraction consisted of endoplasmic reticulum that associated with the crude mitochondrial preparations, but which was absent in preparations of purified mitochondria. The subcellular fractions were assayed for the following marker enzymes; NADPH:cytochrome *c* reductase and glucose-6-phosphate phosphatase (endoplasmic reticulum), cytochrome *c* oxidase (mitochondria), 5'-nucleotidase (plasma membrane), UDP-galactose:N-acetylglucosamine galactosyltransferase (Golgi), and catalase (peroxisomes).

Membrane and soluble fractions of yeast cells were isolated using a French Press lysis procedure. Yeast were grown in CMM (OD₆₀₀ = 0.2-0.8), pelleted by centrifugation (1500 x g, 5 min, 4°C), and washed once with ice-cold lysis buffer (2 mM EDTA, 0.5 mM PMSF, 20 mM HEPES pH 7.5) prior to resuspension in a volume of ice-cold lysis buffer equivalent to 1/15 volume of the original culture. The yeast suspension was then subjected to two successive rounds of lysis in a French Press (2200 pounds per square inch). Non-lysed cells were pelleted by centrifugation (1500 x g, 5 min, 4°C) and the supernatant was collected and centrifuged (30000 x g, 15 min, 4°C) to obtain the total cellular membrane (pellet) and soluble (supernatant) fractions.

Total cellular membranes were isolated from *X. laevis* oocytes according to Scotland *et. al.* (1993). Oocytes (10 per preparation) were suspended in 0.8 ml of ice-cold lysis buffer (1 mM PMSF, 5 mM EDTA, 1 mM MgCl₂, 83 mM NaCl, 5 mM EGTA, 10 mM HEPES pH 7.5) and lysed by Dounce homogenization (20 strokes). The homogenate was centrifuged (800 x g, 5 min, 4°C) and the supernatant was collected and centrifuged again. The resulting supernatant was layered over a 15% (w/v) sucrose pad and the membranes were pelleted by centrifugation (160000 x g, 90 min, 4°C).

Quantitation of the protein content of cellular fractions was performed using the bicinchoninic acid (BCA) assay method originally described by Smith *et. al.* (1985). A 0.1-ml volume of solution containing no protein, protein standard (bovine serum albumin), or unknown protein sample was mixed with a 1.0-ml volume of BCA reagent A:B mixture (1:20, v/v) and incubated at 37°C for 30 min. The absorbance (562 nm) of each mixture was measured and the protein content of the unknown sample was determined from a standard curve.

Expression of MTP-Fusion Proteins in Bacteria

a) Production of *trpE*-MTP Fusion Protein

The procedure of Koerner *et. al.* (1991) was used to express and isolate anthranilate synthase (*trpE*)-MTP fusion protein from UT580 cells. UT580 cells were transformed with a pATH11 vector that contained an in-frame MTP2 cDNA insert (pATHMTP) and plated onto an LB plate that contained tryptophan (20 mg/l) and ampicillin (25 mg/l). A single colony was inoculated into 10 ml of modified M9 medium (6 g/l Na₂HPO₄·H₂O, 3 g/l KH₂PO₄, 0.5 g/l NaCl, 1 g/l NH₄Cl, 5 g/l Bacto-Casamino acids, 20 mg/l tryptophan, 20 g/l glucose, 25 mg/l ampicillin, 3 mM MgSO₄, 0.1 mM CaCl₂) and the culture was grown at 37°C until it reached an OD₆₀₀ of 0.3. The entire culture was then inoculated into 100 ml of modified M9 medium that lacked tryptophan and grown for 2 hr prior to the addition of 0.5 ml of indoleacrylic acid solution (2 mg/ml in 95% ethanol) to induce production of the *trpE*-MTP fusion protein.

The culture was grown for an additional 4 hr and the cells were collected by centrifugation (2500 x g, 5 min, 4°C). The cells were washed once with 10 mM Tris-HCl (pH 7.5), resuspended in 20 ml of buffer (5 mM EDTA, 3 mg/ml hen egg white lysozyme, 50 mM Tris-HCl pH 7.5), and then incubated at 4°C for 2 hr. Cells were lysed by the addition of 1.4 ml of 5 M NaCl and 1.5 ml of 10% (v/v) Nonidet P-40 and further disrupted by sonication with a Braunsonic 1510 sonicator. A portion of the lysed cell mixture (total cell fraction) was removed for storage at -70°C and the remainder of the cell mixture was centrifuged (9000 x g, 10 min, 4°C). The supernatant (soluble fraction) and pellet (insoluble fraction) were recovered and stored at -70°C.

b) Production of *malE-MTP* Fusion Protein

The pMalcR1 vector (New England Biolabs) was used to synthesize maltose-binding (*malE*) fusion protein according to the method of Maina *et. al.* (1988). UT580 cells were transformed with pMalcR1 that contained an in-frame *MTP2* cDNA insert (pMalMTP) and plated. A single colony was inoculated into LB medium that contained 0.2% (w/v) glucose and 25 mg/l ampicillin and grown at 37°C until the culture reached OD₆₀₀ of 0.5, at which point isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.3 mM. The culture was grown for 3 hr and the cells were collected by centrifugation (3000 x g, 5 min, 4°C). Cell pellets were digested with lysozyme and disrupted by sonication, as described above. A portion of the lysed cell mixture (total cell fraction) was removed for storage at -70°C and the remainder of the cell mixture was centrifuged (9000 x g, 10 min, 4°C). The supernatant (soluble fraction) and pellet (insoluble fraction) were recovered and stored at -70°C.

Protein Electrophoresis and Transfer

Protein samples were solubilized in an equal volume of loading buffer (4% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) β-mercaptoethanol, 0.01% (w/v) bromophenol blue, 20 mM Tris-HCl pH 6.8) and subjected to single-dimension SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli (1970). Samples were electrophoretically separated on a slab gel (BioRad Protean II or MiniProtean) using a 4% acrylamide stacking gel and separating gel of 12% acrylamide concentration. Protein was visualized by staining with Coomassie blue solution (0.05% (w/v) Coomassie blue-R250, 25% (v/v) isopropanol, 10% (v/v) acetic acid), followed by destaining in a solution that contained 5% (v/v) methanol and 10% (v/v) acetic acid. Gels were calibrated with one of the following molecular weight standards: (i) Sigma Dalton Mark VII protein standards (α-lactalbumin, 14.2 kDa; trypsin inhibitor, 20.1 kDa; trypsinogen, 24 kDa; carbonic anhydrase, 29 kDa; G-3-P dehydrogenase, 36 kDa; ovalbumin, 45 kDa; bovine albumin, 66 kDa), (ii) BioRad Prestained Low Range SDS-PAGE protein standards (lysozyme, 18.5 kDa; trypsin inhibitor, 27.5 kDa; carbonic anhydrase, 32.5; ovalbumin, 49.5 kDa; bovine serum albumin, 80 kDa; phosphorylase B, 106 kDa), or (iii) Gibco/BRL Prestained 6-60 kDa Protein Ladder (9, 18, 22, 28, 33, 39, 47, 54, 62, and 71 kDa).

Electrophoretically separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore) by semi-dry electrophoretic transfer using an ET-20 Transfer Module (Tyler Research Instruments). Transfers were carried out at 400 mA for 60 min in transfer buffer (180 mM glycine, 20% (v/v) methanol, 0.02% (w/v) SDS, 25 mM Tris-HCl pH 8.3). Membrane blots were air-dried and stored at 4°C.

Generation of Polyclonal Antibodies

a) Antigens

Synthetic Peptides

The amino acid sequence predicted by the L1210/C2 *MTP3* cDNA clone was examined for hydrophilic and charged regions of 15 residues that would constitute

potential immunogenic epitopes. Two 15-amino acid sequences that corresponded to regions in the amino (TFKRSRSDRFYSTRC) and carboxyl (LPSTLPLKHLHSMFC) termini were selected and then synthesized as a conjugate with Diphtheria toxoid by Chiron Mimotopes (Australia).

Additional peptide was synthesized in the laboratory of B. Malcolm (Department of Biochemistry, University of Alberta) with a Rainin PS3 Peptide Synthesizer using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry according to the manufacturer's instructions. The synthesis reaction mixture was washed successively with dimethylformamide and methylene chloride and then dried under vacuum. The peptide was suspended in 5% H₂O/5% thioanisole (v/v) in trifluoroacetic acid (TFA) and incubated, stirring at RT, for 2 hr. The mixture was filtered through a coarse fritted disc and peptide was collected as the filtrate. The TFA was removed from the peptide solution by drying under vacuum at 30°C. Peptide was then precipitated by the addition of ether and washed with ether over a fine fritted disc before being dissolved in 10% acetic acid (v/v) in dH₂O, filtered through a 0.45-µm Millex-GV filter (Millipore), and lyophilized. The molecular weight of the synthesized peptide was determined to be 1962 by mass spectrometry at Synthetic Peptide Incorporated (University of Alberta). This value corresponded closely with the predicted molecular weight value of 1963 for the TFKRSRSDRFYSTRC peptide.

Fusion Protein

The *trpE-MTP* fusion protein was isolated for use as antigen by the following procedure. The insoluble fraction obtained from UT580 cells expressing *trpE-MTP* fusion protein was subjected to electrophoresis on a 12% SDS-polyacrylamide gel. The gel was stained for 10 min with 0.05% (w/v) Coomassie blue R-250 to visualize protein bands. The *trpE-MTP* fusion protein was predicted to be 66.1 kDa based upon the ORF of the pATHMTP vector, and a 66-kDa protein band (which was absent in the insoluble fraction obtained from UT580 cells transformed with the vector that expressed only *trpE* protein) was excised from the gel. The *trpE-MTP* protein was eluted from the gel slice by electroelution (12-kDa molecular weight cutoff membrane, BioRad Electroelution apparatus) according to the manufacturer's instructions. The protein eluate was concentrated by centrifugation through a Millipore Ultrafree MC spin cartridge (10 kDa molecular weight cutoff, 2000 x g). The protein solution was recovered, diluted in half with PBS, and stored at -20°C.

b) Production of Polyclonal Antibodies

Polyclonal antibodies were raised by injection of antigen into New Zealand White rabbits according to established procedures (Harlow and Lane, 1988). The injections, bleedings, and animal handling were performed by Health Sciences Laboratory Animal Services personnel (University of Alberta). All blood samples collected were allowed to stand at RT for 3-4 hr, then overnight at 4°C. Blood samples were centrifuged (500 x g, 5 min) and serum was collected and aliquoted for storage at -20°C. For immunization, the rabbits received a primary sub-cutaneous injection (into the inter-scapular region) of antigen (≈ 0.2 mg peptide-Diphtheria toxin conjugate or fusion protein) homogenized (1:1, v/v) with Freund's complete adjuvant. About 30-40 days later, and at 7-9 day intervals twice thereafter, rabbits were injectioned with antigen homogenized (1:1, v/v) with Freund's incomplete adjuvant. Blood (5 ml) was collected by ear bleeding prior to each injection and immune serum was prepared by centrifugation as described above. Rabbits were anaesthetized at the end of the injection schedule and terminated by lethal injection.

c) Immunopurification of Polyclonal Antibodies

Antiserum produced against *trpE-MTP* fusion protein was enriched for antibodies specific for the *MTP*-encoded protein according to a method described by Sambrook *et al.*

al. (1989). The insoluble UT580 cell fraction containing *malE-MTP* fusion protein was subjected to SDS-PAGE on a 12% acrylamide gel. The electrophoretically separated proteins were transferred to a PVDF membrane which was then incubated in a solution of 1 mg/ml Ponceau S in 5% acetic acid to visualize protein bands. The *malE-MTP* fusion protein was predicted to be 72 kDa based upon the ORF of the pMalMTP vector, and a region of membrane that contained a 68-kDa protein band (which was absent in the insoluble fraction obtained from UT580 cells transformed with the vector that expressed only *malE* protein) was excised. The Ponceau S stain was removed by washing in dH₂O and the membrane was incubated (RT, 60 min) in PBS that contained 3% BSA and then at 4°C for 12 hr with antiserum (1/50 volume) raised against the *trpE-MTP* fusion protein. The membrane was washed (20 min, RT) successively in 0.15 M NaCl and PBS and then incubated in a small volume of elution buffer (1 mM EGTA, 0.2 M glycine pH 2.8) for 20 min at RT. The elution buffer, which contained antibodies against the fusion protein antibody (α -F), was collected and mixed with a volume of 1 M Tris-HCl (pH 7.4) and PBS (10X concentration) equivalent to 1/10 the volume of the elution buffer. The purified antibodies were stored in aliquots at -20°C.

Antibodies produced against the peptide-Dipteria toxin conjugate were purified by affinity chromatography on peptide-sepharose columns by an established method (Harlow and Lane, 1988). The peptide-sepharose matrix was prepared from CNBr-activated sepharose according to the manufacturer's instructions (Pharmacia). Briefly, 0.5 g of CNBr-activated sepharose was washed extensively in 1.0 M HCl and suspended in coupling buffer (0.5 M NaCl, 0.1 M NaHCO₃ pH 8.3). Approximately 10 mg of peptide (TFKRSRSDRFYSTRC) in coupling buffer was combined with the sepharose solution and incubated for 2 hr at RT with constant mixing. The peptide-sepharose matrix was washed twice with coupling buffer and incubated in 1.0 M ethanolamine (pH 10) for 2 hr at RT with constant mixing. The matrix was washed successively (three times each) in 0.5 M NaCl/0.1 M sodium acetate (pH 4), 0.5 M NaCl/0.1 M Tris-HCl (pH 8), and Tris-buffered saline (TBS; 0.5 mM NaCl, 20 mM Tris-HCl pH 7.5). A 1.5-ml bed volume of peptide-sepharose matrix was poured into a column (0.6 x 5.0 cm) which was stored at 4°C in TBS that contained 0.02% sodium azide. The peptide-sepharose column was sequentially washed with 4 column volumes of (i) 10 mM Tris-HCl (pH 7.5), (ii) 0.1 M glycine (pH 2.5), (iii) 10 mM Tris-HCl (pH 8.8), (iv) 0.1 M triethylamine (pH 11.5), and (v) 10 mM Tris-HCl (pH 7.5). A 0.5-ml portion of peptide antiserum was diluted to 5.0 ml with 10 mM Tris-HCl (pH 7.5) and passed through the column three times. The column was then washed with 4 column volumes of 10 mM Tris-HCl (pH 7.5) and a solution of 0.5 M NaCl/10 mM Tris-HCl (pH 7.5). Antibodies were eluted at low pH with 4 column volumes of 0.1 M glycine (pH 2.5) into a tube that contained 1/2 column volume of 1.0 M Tris-HCl (pH 8). To remove any remaining antibodies, the column was washed with 4 column volumes of 10 mM Tris-HCl (pH 8.8) and then eluted at high pH with the addition of 4 column volumes of 0.1 M triethylamine (pH 11.5). The low and high pH antibody-containing eluates were combined and mixed with a 1/10 volume of 1% (w/v) BSA solution. The resulting affinity purified peptide antibody (α -P) solution was stored in aliquots at -20°C.

Immunostaining (Western) Analysis

Immunostaining was performed by the following method and all incubations and washes were at 4°C on a shaking platform, unless otherwise stated. PVDF membrane blots were (i) washed for 30 min in Tween-TBS (TTBS; TBS containing 0.2% (v/v) Tween-20), (ii) then blocked by incubating at least 6 hr in TTBS containing 5% (w/v) skim milk powder, and (iii) washed twice with TTBS (15 min per wash). The blots were incubated for $\geq$ 12 hr in TTBS that contained 1% (w/v) skim milk powder and primary antibodies, after which they were washed three times with TTBS (15 min each wash) and

incubated for 2-3 hr in TTBS that contained 1% skim milk powder (w/v) and secondary antibodies (goat anti-rabbit horse-horse radish peroxidase (HRP) conjugate; 1:3000 dilution (v/v)). The blots were washed three times (15 min each wash) with TTBS and TBS and then antibody-antigen complexes were detected with the enhanced chemiluminescence (ECL) reagent detection kit (Amersham Life Sciences), according to the manufacturer's instructions. Briefly, the blots were overlaid with a solution consisting of 1:1 (v/v) detection solution 1:detection solution 2, shaken to remove excess detection solution, placed between acetate sheets and exposed to Kodak XAR-5 film.

Immunofluorescence Cell Staining

Adherent cultures of mammalian cells were subjected to indirect immunofluorescence staining using a modification of the method of Harlow and Lane (1988). Cells grown on circular glass coverslips (Dynalab) were fixed by incubation (10 min, RT) in 3% (w/v) paraformaldehyde in PBS, washed in PBS (5 min, RT), and then permeabilized by incubation (5 min, RT) in PBS that contained 0.5% (v/v) Triton-X100. The coverslips were washed in PBS (5 min, RT) and incubated with primary antibody (that had been diluted 1/25-1/50th (v/v)) in PBS at 37°C for 30 min. Following three successive washes in PBS (5 min each, RT), the coverslips were incubated in PBS that contained anti-rabbit IgG-rhodamine B (1:300 dilution (v/v)) at 37°C for 30 min. The coverslips were washed three times with PBS (5 min each, RT) and mounted in glycerol-gelatin (Sigma) onto microscope slides.

Immunofluorescence staining of *S. cerevisiae* was performed according to Harlow and Lane (1988). Yeast cells were collected by centrifugation (2500 x g, 5 min) and (i) washed once with sorbitol buffer (1.2 M sorbitol, 0.12 M K₂HPO₄, 33 mM citrate pH 5.9), (ii) fixed by incubation (30 min, RT) in 4% (w/v) paraformaldehyde in sorbitol buffer, (iii) washed twice in sorbitol buffer (RT) and (iv) suspended and incubated (30 min, 30°C) in sorbitol buffer that contained 8 mg/ml Zymolyase 20000 (Kerin Brewery). The resulting sphaeroplasts were collected by centrifugation (500 x g, 1 min) and resuspended in sorbitol buffer. Portions (10 µl) of the suspension were incubated (15 min, RT) on poly-L-lysine coated coverslips to immobilize sphaeroplasts for immunofluorescence staining. The coverslips were (i) sequentially incubated in methanol (-20°C, 2 min) and acetone (-20°C, 30 sec), (ii) washed twice (5 min each, RT) in PBS that contained 1% (w/v) BSA, (iii) incubated (37°C, 30 min) with purified α-F (1/50 dilution (v/v)) in PBS that contained 2% (w/v) BSA, (iv) washed three times (5 min each, RT) in PBS, (v) incubated with anti-rabbit IgG-rhodamine B (1/300 dilution, (v/v)) in PBS at 37°C for 30 min, (vi) washed three times with PBS (5 min each, RT) and (vii) mounted with glycerol-gelatin onto microscope slides.

Fluorescence microscopy was performed using a Zeiss Axioskop 20 microscope equipped with NeoFluar objective lens (40x or 63x) and a MC100 camera. Photographs were obtained using Kodak T_{max} 400 film with a camera setting of ASA 1600.

Methods for Manipulation of DNA

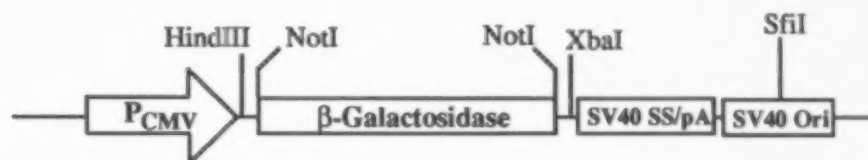
All procedures used for the manipulation of DNA are described in detail in the Methods and Materials section of Chapter III and included preparation of plasmid DNA, enzymatic manipulations, and PCR reactions and DNA sequencing. *In vitro* transcription from cDNA inserts that had been directionally cloned behind a T7 RNA promoter were performed using the T7 MessageMachine Transcription kit (Ambion Inc.) according to the manufacturer's instructions.

Construction of Expression Vectors

The vectors employed in this study and their method of construction are listed below. A partial linear map indicating relevant regions of the vector and landmark restriction enzyme sites accompanies the description of each expression vector (maps are

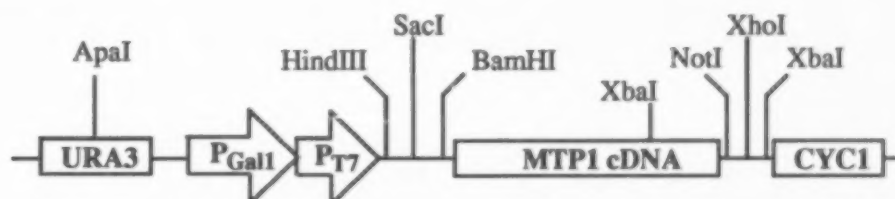
not to scale). The cDNA-containing vectors were DNA sequenced to confirm their identity using oligonucleotide primers that corresponded to regions of the vector which flanked the cDNA insert. Abbreviations are: *trpE*, *E. coli* anthranilate synthase gene; *malE*, *E. coli* maltose-binding protein gene; *P_{tac}*, *E. coli tac* promoter; *P_{trp}*, *E. coli trp* operon promoter; *rrnB*, yeast *rrnB* transcriptional terminator; β -lac, *E. coli* β -lactamase gene; *CUP1*, yeast copper metallothionein promoter; *CYC1*, yeast *CYC1* transcriptional terminator; *URA3*, orotidine-5'-P-decarboxylase gene; PCMV, Cytomegalovirus promoter; Ori, origin of replication; SV40 SS/pA, SV40 splice site and polyadenylation signal; *P_{Gall}*, yeast galactokinase promoter; *P_{T7}*, T7 bacteriophage RNA polymerase promoter.

a) pcDNA1-AMP- β Gal



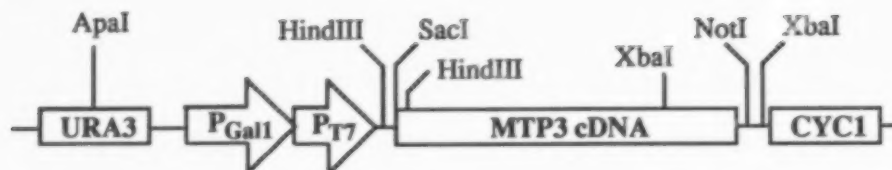
The β -galactosidase gene was removed from the vector pCMV β Gal as a 3.2-kbp NotI fragment and cloned into the vector pcDNA1-AMP to yield pcDNA1-AMP- β Gal. pcDNA1-AMP is a mammalian expression vector that contains the CMV promoter and enhancer, the SV40 and Polyoma virus origins of replication, and the β -lactamase gene. The correct orientation of the β -galactosidase gene in pcDNA1-AMP- β Gal was confirmed by the capacity of the vector to mediate β -galactosidase activity in transfected Cos-1 cells.

b) pMTP1



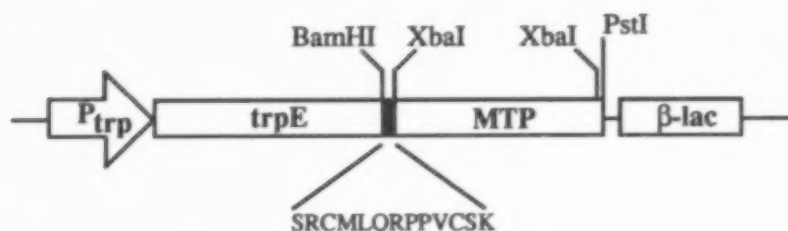
The plasmid pMTP1 was isolated during a functional complementation screen of a L1210/C2 cDNA library in yeast, as described in Chapter III. The library vector was pYES2, a high copy yeast/*E. coli* shuttle plasmid carrying the *URA3* auxotrophic selectable marker, *Gall* and T7 promoters, and the *CYC1* transcriptional terminator. The *MTP1* cDNA encompassed nucleotides 384-1435 of the *MTP3* cDNA sequence shown in Fig. IV 1.

c) pMTP3



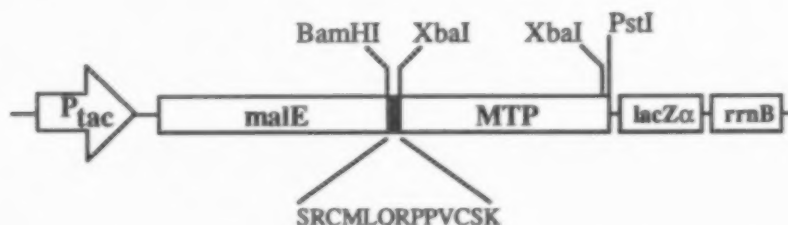
The *MTP3* cDNA encompasses the entire 1435-nucleotide cDNA sequence of *MTP3* (Fig. IV 1) and was cloned into the pYES2 vector as described in the Methods and Materials section of Chapter III.

d) pATHMTP



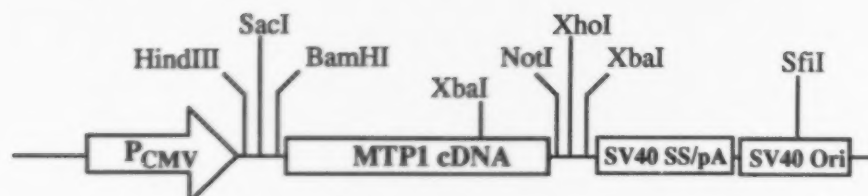
The *MTP2* cDNA, which encompasses nucleotides 88-1435 of the sequence shown in Fig. IV 1, was subcloned as a (5') BamHI-*MTP2* cDNA-PstI (3') fragment from the pT7-6-MTP vector (described in the construction of pMTP2, Chapter III) into the *E. coli* expression vector pATH11 (Koerner *et. al.*, 1991). The pATH11 vector is a high copy plasmid that carries a β -lactamase gene and the inducible *E. coli* *trp* operon promoter that mediates the transcription of the *trpE* fusion genes. The resulting vector pATHMTP contained an in-frame ligation of *MTP* cDNA with the *trpE* gene. An additional region coding for 13 amino acids (SRCMLQRPPVCSK) was between the *trpE* and *MTP* coding regions, a result of the subcloning of the polylinker region of pT7-6 along with the cDNA sequence.

e) pMalMTP



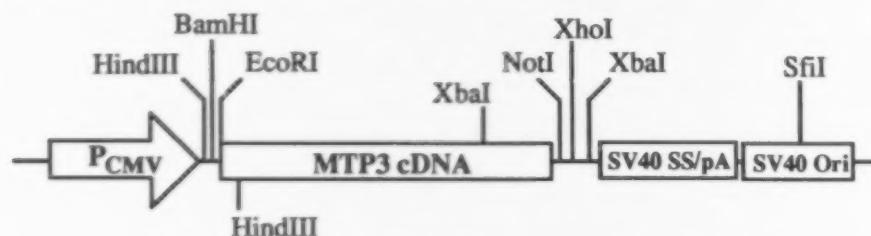
The *E. coli* expression vector pMalcRI (New England Biolabs) is a low copy plasmid that expresses the *malE* gene under transcriptional control of an IPTG-inducible *tac* promoter and contains the β -lactamase and *lacI* genes. The (5') BamHI-*MTP2* cDNA-PstI (3') fragment of pATHMTP was cloned in-frame with the *malE* gene to generate pMalMTP. The vector pMalMTP contained an additional 13 amino acids (SRCMLQRPPVCSK) between the *malE* and *MTP* coding regions as a result of the subcloning of the polylinker region of pT7-6 along with the cDNA sequence.

f) pcDMTP1



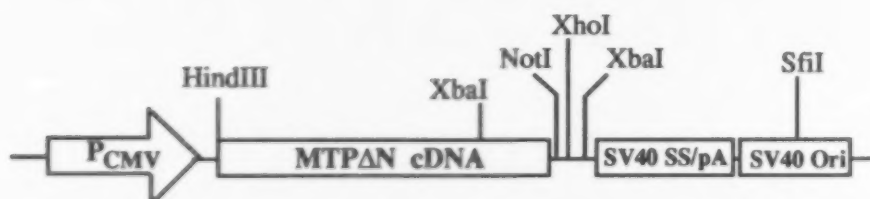
The pcDMTP1 vector was constructed by the direct subcloning of the (5') HindIII-*MTP1* cDNA-NotI (3') fragment of pMTP1 into the pcDNA1-AMP vector (Invitrogen Corp.). The pcDNA1-AMP vector is a mammalian expression vector that contains the CMV promoter, the SV40 splice site and polyadenylation signal, the T7 RNA polymerase promoter and the Polyoma virus origin of replication.

g) pcDMTP3



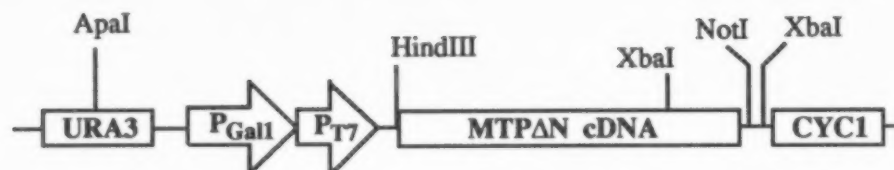
The (5') SacI-*MTP3* cDNA-XhoI (3') fragment of pMTP3 was cloned into the pMTL22 vector (Chambers *et al.*, 1988), and then subcloned as the (5') EcoRI-*MTP3* cDNA-XhoI (3') fragment into pcDNA1-AMP. The resulting vector, pcDMTP3, contained the entire *MTP3* cDNA sequence.

h) pcDMTPΔN



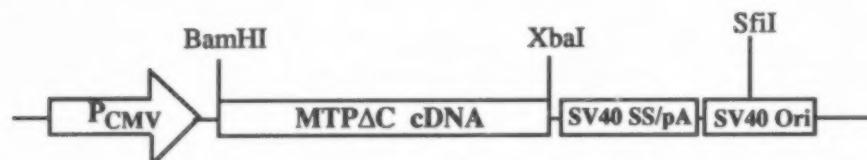
An amino terminus-truncated version of the *MTP3* cDNA sequence was generated by PCR amplification of pMTP3 using primers that corresponded to the *CYC1* terminator (5' GCGTGAATGTAAGCGTG 3') of pYES2 and an internal region of the *MTP3* cDNA (5' GGCTCTATAAGCTTGCCACCATGGTCCGCACCGGGACGA 3', HindIII site underlined). The (5') HindIII-*MTPΔN* cDNA-NotI (3') PCR fragment was directly cloned into pcDNA1-AMP to generate the vector pcDMTPΔN. The coding region of the *MTPΔN* cDNA encompassed nucleotides 208-1435 (lacked the nucleotides encoding the first 24 amino acids) shown in Fig. IV 1 and contained a consensus sequence for initiation of translation (5' GCCACCATGG 3', initiating ATG underlined) according to Kozak (1991).

i) pMTP Δ N



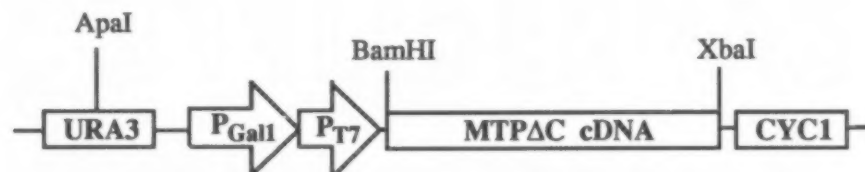
The pMTP Δ N vector was created by directly cloning the (5') HindIII-*MTP* Δ N cDNA-NotI (3') fragment of pcDMTP Δ N into pYES2.

j) pcDMTP Δ C



A carboxyl terminus-truncated version of the *MTP3* cDNA sequence was generated by PCR amplification of pcDMTP3 using primers that corresponded to the T7 promoter region (5' TAATACGACTCACTATAGGG 3') of pcDNA1-AMP and an internal region of the *MTP3* cDNA (5' GGCTCTATTCTAGACTATCTCTGGCACATT TCTGTTG 3', XbaI site underlined). The (5') BamHI-*MTP* Δ C cDNA-XbaI (3') PCR fragment was directly cloned into pcDNA1-AMP to generate the vector pcDMTP Δ C. The coding region of the *MTP* Δ C cDNA encompassed nucleotides 1-730 (lacked the final 21 amino acids) shown in Fig. IV 1 and contained a translational stop codon (TAG).

k) pMTP Δ C



The pMTP Δ C vector was created by directly cloning the (5') BamHI-*MTP* Δ C cDNA-XbaI (3') fragment of pcDMTP Δ C into pYES2.

RESULTS

Production and Characterization of *MTP*-Specific Polyclonal Antibodies

Immunological probes were used to identify *MTP* and characterize its cellular location. To ensure the production of *MTP*-specific antibodies, two alternate sources of antigen were used: (i) a bacterially expressed *trpE*-*MTP* fusion protein, and (ii) a synthetic peptide corresponding to a potentially immunogenic region of *MTP*.

The pATH vectors are a series of plasmids that contain (i) an inducible *E. coli trp* operon promoter upstream of an *E. coli* anthranilate synthase (*trpE*) gene, and (ii)

different multiple cloning sites following the *trpE* gene that provide for in-frame fusion of cDNA sequences (Koerner *et al.*, 1991). In the absence of tryptophan, transcriptional attenuation of the *trp* promoter is relieved and high-level transcription and translation occurs. To produce *MTP* for use as antigen, nucleotides 88-1049 of the *MTP3* cDNA sequence (Fig. IV 1; this cDNA sequence encompassed most of the 5' untranslated region that contained no potential stop codons) were inserted in-frame with the *trpE* gene in the pATH11 vector to create the pATHMTP vector. The ORF of the *MTP* cDNA in the pATH 11 vector, which includes both the 5' untranslated region and predicted ORF, should encode a 29-kDa protein. Since the *trpE* gene encodes a 37-kDa protein, the *trpE-MTP* fusion gene was expected to be expressed as a 66-kDa fusion protein. The pATH11 and pATHMTP vectors were transformed into *E. coli* and transformed cells were induced to express the *trpE* and *trpE-MTP* fusion proteins as described in Methods and Materials. The proteins present in the total cell lysates and the insoluble cell fractions of pATH11- and pATHMTP-transformed cells were separated by SDS-PAGE and visualized by Coomassie blue staining, as shown in Panel A of Fig. IV 2. A 37-kDa protein band was found primarily in the insoluble fraction of pATH11-transformed cells and was absent in the fractions from pATHMTP-transformed cells, indicating the expression of the *trpE* protein in pATH11 but not pATHMTP-transformed cells. As predicted, the *trpE-MTP* fusion protein was observed to migrate in a 66 kDa protein band in the insoluble fraction of pATHMTP-transformed cells. This 66-kDa protein band was eluted from SDS-polyacrylamide gels and used as antigen to produce polyclonal antibodies against the *trpE-MTP* fusion protein. Additional protein bands of approximately 48 and 52 kDa were present in the insoluble fraction of pATHMTP-transformed cells and were absent in the insoluble fraction of pATH11-transformed cells. Presumably these smaller proteins resulted from premature termination of transcription/translation and/or were degradation products of the 66-kDa *trpE-MTP* fusion protein.

A second *MTP* fusion protein was produced for use in the purification and characterization of polyclonal antibodies raised against the *trpE-MTP* fusion protein. Nucleotides 88-1049 of the *MTP3* cDNA sequence (shown in Fig. IV 1) were ligated in-frame behind the *malE* (maltose binding protein) gene in the pMalcR1 vector (Maina *et al.*, 1988) to create the pMalMTP vector. Expression of the 42-kDa *malE* protein and the predicted 71-kDa *malE-MTP* fusion protein was under control of the IPTG-inducible *E. coli* *tac* promoter. Soluble and insoluble cell fractions were isolated from IPTG-induced cells carrying pMalcR1 or pMalMTP, and the proteins were separated by SDS-PAGE and stained with Coomassie blue (Fig. IV 2, Panel B). A protein band of ~68 kDa was observed in the insoluble fraction of pMalMTP-transformed cells and was absent in either the soluble or insoluble fractions of pMalcR1-transformed cells, suggesting the presence of the *malE-MTP* fusion protein in this band. The insoluble fraction of pMalMTP-transformed cells was subjected to SDS-polyacrylamide electrophoresis and transferred to a PVDF membrane, whereupon the region of the membrane that contained the 68-kDa *malE-MTP* fusion protein was excised and used to purify the anti-fusion protein antibody by immunoadsorption.

The predicted amino acid sequence of the *MTP3*-encoded protein (Fig. IV 1) was examined for the presence of a potentially antigenic 15 amino acid region for use in the generation of polyclonal antibodies. A highly charged region corresponding to amino acid residues 5-19 (TFKRSRSDRFYSTRC) was selected and the corresponding synthetic peptide was used to produce anti-peptide (α -P) antibodies as described in Methods and Materials. A second synthetic peptide, corresponding to a region near the carboxyl terminus of *MTP3* (amino acid residues 199-213, LPSTLPLKHLHSMFC), was synthesized but failed to produce immuno-reactive antibodies upon injection into rabbits (not shown).

The immunoreactive specificity of the α -P antibodies was demonstrated by western analysis of extracts prepared from UT580 cells that either expressed or did not express one of the two fusion proteins. Panel A of Fig. IV 3 illustrates that the α -P antibodies recognized the *MTP* fusion proteins in the insoluble fractions prepared from cells transformed with either the pATHMTP or pMalMTP vectors. There was little, if any, cross-reactivity of α -P antibodies with other proteins. The specificity of the α -P antibodies for the peptide epitope in the *MTP* fusion protein was demonstrated by conducting western blot analyses with antibodies that were first incubated in the absence or presence of excess peptide (TFKRSRSDRFYSTRC). Panel B of Fig. IV 3 shows that the antibodies incubated with excess peptide were unable to recognize the *malE-MTP* fusion protein, thereby confirming that its immunoreactivity was epitope-specific.

The ability of the α -F antibodies to recognize *MTP* is shown in Fig. IV 4. The α -F antibodies specifically reacted with the 66- and 68-kDa *MTP* fusion proteins of the insoluble fractions prepared from UT580 cells transformed with either the pATHMTP or pMalMTP vectors, respectively. The absence of immunoreactivity with similar-sized proteins in cell fractions prepared from cells expressing only the *trpE* or *malE* proteins indicated that the α -F antibodies recognized *MTP* although there was cross-reactivity with a number of other proteins. These smaller proteins likely resulted from premature termination of transcription/translation and/or were degradation products of the *MTP* fusion proteins.

Expression of *MTP* cDNAs in Yeast

The antibodies were used to assess expression of the *MTP1* cDNA-encoded protein in KTK cells transformed with pMTP1 by western blot and immunofluorescence analysis. Crude membrane and soluble cell fractions were prepared from pMTP1-transformed KTK cells that had been grown in the presence of methotrexate, sulfanilamide, and 100 μ M thymidine, and, as a control, from pYES2-transformed KTK cells that had been grown under identical conditions except that 1 mM thymidine was utilized to allow cell survival and growth. Analysis of cell fractions using the α -F antibodies indicated the presence of a $\approx$ 16-kDa immunoreactive protein in the membrane fraction of pMTP1-transformed cells that was absent in pYES2-transformed cells (Fig. IV 5, Panel A). The α -F antibodies were also used for immunofluorescence staining of cells grown under the identical conditions (Panel B, Fig. IV 5). A general staining over the entire cell surface was observed with cells transformed with pMTP1, but not with cells transformed with pYES2. These results indicated that the *MTP1*-encoded protein was expressed in the plasma membranes of KTK cells transformed with pMTP1.

The expression of the complete protein, encoded by the *MTP3* cDNA, was assessed under conditions identical to those used to localize the *MTP1*-encoded protein in the plasma membranes of transformed cells. KTK cells were transformed with the pMTP3 vector and plated onto medium that contained methotrexate, sulfanilamide and 100 μ M thymidine. However, as illustrated in Panel A of Fig. IV 6, pMTP3-transformed KTK cells did not survive; under these conditions, pMTP1-transformed cells survived and formed colonies. This result suggested that either the protein encoded by the *MTP3* cDNA did not mediate thymidine salvage or it was not expressed in the plasma membrane of KTK cells. To determine if KTK cells could express the *MTP3*-encoded protein, membrane and soluble cell fractions were prepared from pYES2-, pMTP1- and pMTP3-transformed KTK cells and subjected to western blot analysis (Fig. IV 6, Panel B). The α -F antibodies again recognized a $\approx$ 16-kDa protein in membranes of pMTP1-transformed cells and not in membranes of pYES2-transformed cells. However, no immunoreactive proteins were observed in either the soluble or membrane fractions of

cells transformed with pMTP3. To confirm this observation, an identical blot was probed with α -P antibodies which, because of their epitope specificity, should recognize the *MTP3*-encoded protein and not the *MTP1*-encoded protein. As illustrated in Panel B of Fig. IV 6, the *MTP3*-encoded protein was not detected by the α -P antibodies. These results suggested that the protein encoded by *MTP3* cDNA was not expressed in yeast, thereby explaining the inability of pMTP3-transformed cells to survive in the presence of methotrexate, sulfanilamide, and 100 μ M thymidine.

Expression of *MTP* cDNAs in *Xenopus laevis* Oocytes

Functional expression of foreign proteins in *X. laevis* oocytes, by injection of complementary RNA (cRNA) has proven useful in the identification and characterization of numerous cDNAs encoding mammalian plasma membrane proteins (For review, see Sigel, 1990). Since the full length *MTP* was not expressed in pMTP3-transformed yeast, the ability of the *MTP1*- and *MTP3*-encoded proteins to mediate thymidine uptake was examined by functional expression in *X. laevis* oocytes. *MTP1* and *MTP3* cRNAs were transcribed *in vitro* by standard methods and injected into oocytes. As controls, oocytes were injected with H₂O. Uptake of 100 μ M radiolabeled substrates by cRNA- and H₂O-injected oocytes was measured 2 to 3 days later. The injection of *MTP1* cRNA into oocytes (Fig. IV 7, Panel A) resulted in a 2-3 fold increase in permeability to thymidine, uridine and sucrose relative to that observed with the H₂O-injected control oocytes. There was little, if any, difference in adenosine permeability between *MTP1* cRNA-injected and H₂O-injected oocytes. An approximate 30% decrease in leucine uptake was observed in *MTP1* cRNA-injected oocytes relative to H₂O-injected oocytes. In a series of identical experiments that were conducted with oocytes that had been injected with *MTP3* cRNA (not shown), there were no differences in uptake of any of these substrates between *MTP3* cRNA- and H₂O-injected oocytes. The results of these functional assays suggested that expression of the truncated *MTP1*-encoded protein resulted in an altered permeability of *Xenopus* oocytes to low molecular weight organic solutes.

The inability of the *MTP3*-encoded protein to alter permeability in oocytes may have been due to its lack of expression, as was observed in pMTP3-transformed yeast. Therefore, western blot analyses were performed to determine if the *MTP1*- and *MTP3*-encoded proteins were present in crude membrane fractions isolated from oocytes injected with *MTP1* or *MTP3* cRNA. These analysis, which were conducted with the α -F and α -P antibodies, are shown in Panel B of Fig. IV 7. The α -F antibodies recognized a single band ($\approx$ 16 kDa) in the membranes of *MTP1* cRNA-injected oocytes and two bands ($\approx$ 18 and $\approx$ 23.5 kDa) in the membranes of *MTP3*-injected oocytes. The detection of this additional 18-kDa protein in *MTP3* cRNA-injected oocytes by the α -F antibodies suggested that the oocytes recognized an alternate translational start codon downstream of the predicted ATG start codon. The presence of a $\approx$ 23.5-kDa *MTP3*-encoded protein was demonstrated by its immunoreactivity with the α -P antibodies, which by virtue of their epitope specificity, failed to recognize any immunoreactive proteins in *MTP1* cRNA-injected oocytes. The *MTP3*-encoded protein identified by the α -P antibodies exhibited an identical molecular weight ($\approx$ 23.5 kDa) to that observed for the *in vitro* translation product of *MTP3* cDNA (Fig. III 10). There were no immunoreactive proteins present in the membranes of H₂O-injected oocytes.

The results obtained in the oocyte expression studies demonstrated directly that the *MTP1*-encoded protein induced thymidine influx. However, the failure of the full length *MTP3*-encoded protein to stimulate influx of thymidine suggested that *MTP* either (i) did not directly stimulate thymidine flux, or (ii) was capable of stimulating thymidine flux but was not localized to the plasma membrane. To address the latter possibility,

western blot and immunofluorescence staining analyses were performed on mammalian cells to determine the subcellular location of *MTP*.

Expression of *MTP* in Cultured Cells

Since yeast were unable to express the full length *MTP3* cDNA-encoded protein and expression of this protein in *Xenopus* oocytes did not induce thymidine uptake, the expression of this protein was examined in cultured cells. The pcDMTP3 vector, which contained the *MTP3* cDNA sequence, was transfected into cultured Cos-1 cells using a DEAE-dextran method and the expression of *MTP3* protein was assessed by western blot analysis of nucleus-free ("post-nuclear") cellular extracts. As a control, transfections using the pcDNA1-AMP-CAT vector were performed under identical conditions. Immunostaining with the α -P antibodies revealed a band (≈ 23.5 kDa) that was clearly evident in post-nuclear membrane fractions of pcDMTP3-transfected cells and barely visible in the same fractions isolated from CAT-transfected cells (Fig. IV 8, Panel A). The ≈ 23.5 kDa band was absent in immunoblots prepared with α -P antibodies that had been pre-incubated with excess peptide (TFKRSRSDRFYSTRC). Additional immunoreactive material (≥ 65 kDa) was observed in the post-nuclear soluble fractions of pcDMTP3- and CAT-transfected cells, however, these bands represented background staining since they were present in the immunoblots prepared with α -P antibodies that had been pre-incubated with excess peptide. These results suggested that *MTP3*-encoded polypeptides were expressed in large quantities in cells transfected with pcDMTP3.

The electrophoretic characteristics of the immunoreactive material produced by pcDMTP3-transfected cells were examined in greater detail by measurement of mobilities under reducing and non-reducing conditions and in conjunction with heat denaturation (Fig. IV 8, Panel B). In non-reduced samples, the ≈ 23.5 -kDa band and a faint ≈ 45 -kDa band of immunoreactive material were observed. The disappearance of the ≈ 45 -kDa band and the increase in signal intensity of the ≈ 23.5 -kDa band observed in samples subjected to reduction with β -mercaptoethanol suggested that aggregation of *MTP3*-encoded polypeptides had occurred under non-reducing conditions. Heat denaturation of samples did not alter the electrophoretic characteristics of *MTP3*-encoded polypeptides.

Upon close inspection of the immunoblots shown in Panel B of Fig. IV 8, a very faint signal of ≈ 23.5 kDa was evident in post-nuclear membranes of Cos-1 cells that had been transfected with the control plasmid. The presence of the signal suggested expression of *MTP* in post-nuclear membrane fractions of non-transfected cells at low levels. To assess the relative abundance of *MTP* or related proteins, within and between cell types, post-nuclear membrane fractions were isolated from L1210/C2, S49, HeLa, and Cos-1 cells and examined for the presence of immunoreactive polypeptides recognized by α -P antibodies. The immunoblot shown in Fig. IV 9 was purposely over-exposed in an attempt to visualize immunoreactive material. A very faint signal of ≈ 23.5 kDa was present in membranes prepared from Cos-1 cells and absent in membranes from L1210/C2, S49, or HeLa cells. The additional immunoreactive bands present in membranes prepared from all cells likely represent background or artifact staining. The presence of a faint 23.5-kDa signal in non-transfected Cos-1 cells and absence of a similar sized signal in L1210/C2, S49, or HeLa cells suggested that *MTP*, or a closely related protein, may be expressed at barely detectable or non-detectable levels in post-nuclear membrane fractions.

The lack of a ≈ 23.5 -kDa immunoreactive band in post-nuclear membranes of L1210/C2 cells was surprising because the *MTP* cDNA was isolated from a cDNA library that had been prepared from polyA⁺ RNA isolated from this cell line. Since preparations of post-nuclear membranes contain all cellular membranes except those of the nucleus, this lack of immunoreactivity may have resulted from a "dilution" of *MTP*-containing

membrane with total cellular membranes. In an attempt to demonstrate the presence of *MTP* in the plasma membrane of L1210/C2 cells, the cell line from which the *MTP* cDNA was isolated, enriched plasma membrane fractions were prepared by the Percoll centrifugation method of Hogue *et al.* (1991) and examined for the presence of proteins recognized by antibodies specific for *MTP*. Subjected to SDS-PAGE, the proteins were transferred to PVDF membranes and probed with α -P and α -F antibodies. An absence of immunoreactive material was observed during repeated analyses of enriched plasma membrane fractions², suggesting that *MTP* was not localized in the plasma membrane of L1210/C2 cells.

An *MTP*-Related Protein is Present in an Intracellular Membrane Compartment of Rat Hepatocytes

MTP did not appear to be present in the plasma membranes of L1210/C2 cells, thus the protein may have been localized to a specific intracellular membrane. As discussed in Chapter III, the nucleotide and predicted amino acid sequences of the *MTP* cDNA and its human homolog (Momura *et al.*, 1993) are 92% and 97% identical, respectively, suggesting that *MTP* and related proteins in other species may be highly conserved. This high degree of conservation predicted that antibodies raised against *MTP* may also recognize these related proteins in other species. This cross-reactivity would therefore provide a means to identify *MTP*-related protein in cells from species other than mouse. Based upon this predicted cross-reactivity, and the observation that a *MTP*-related RNA transcript exists in rat Walker 256 cells (Fig. III 7, Chapter III), the possible expression of *MTP*-related protein was examined by immunoblotting against subcellular membrane fractions prepared from rat liver cells by a well characterized method (Vance and Vance, 1988; Vance, 1990). These fractions were obtained from J. Vance (University of Alberta) and included total cellular membranes, enriched preparations of Golgi and (smooth and rough) endoplasmic reticulum, crude and purified preparations of mitochondria and mitochondrial-associated membranes. Western blot analysis was performed on subcellular membrane fractions that contained equal quantities of protein (Fig. IV 10). A band of immunoreactive material that migrated at ≈ 23.5 kDa was present only in the Golgi-enriched membrane fraction, suggesting that a rat homolog of *MTP* is localized to the Golgi compartment.

Localization of Transiently-Expressed *MTP3* in Cos-1 Cells by Immunofluorescence Staining

In an attempt to determine the location of *MTP*-encoded protein in mammalian cells, indirect immunofluorescence staining was performed on pcDMTP3-transfected Cos-1 cells which had previously been demonstrated to express high levels of *MTP3*-encoded protein. In the experiments of Figs. IV 11 and 12, Cos-1 cells expressing recombinant *MTP3* were examined at periods of 22, 28, and 72 hrs after transfection, and as controls, immunofluorescent staining was conducted on cells that had been transfected with the pcDNA1-AMP-CAT vector. These staining experiments used cell cultures that had been grown on glass coverslips and $\approx 90\%$ of the plated cells were observed to dislodge from the coverslips as a result of manipulation during the staining procedure. Thus, the results of these experiments represent staining of $\approx 10\%$ of the original numbers of cell plated on the coverslips. At 22 hr after transfection (Fig. IV 11), about 20% of the cells that had been transfected with pcDMTP3 displayed a faint fluorescence in a centralized intracellular region and many of these cells exhibited a more intense

² Western blot analysis of enriched plasma membrane fractions prepared from L1210/C2 cells was performed on approximately ten different occasions using four different plasma membrane preparations. The quantity of material subjected to SDS-PAGE in these experiments ranged from 25-250 μ g protein per sample.

fluorescence in the peripheral region of the nucleus (Fig. IV 11, Bottom Panel). This pattern of fluorescence was absent in (i) a major portion ($\approx 80\%$) of the pcDMTP3-transfected cell population and (ii) the pcDNA1-AMP-CAT transfected cell population.

Examples of immunofluorescent staining of a pcDMTP3-transfected cell culture performed at 48 and 72 hr after the transfection procedure are illustrated in Fig. IV 12. At 48 hrs after transfection, a minor portion of the cell population was observed to exhibit fluorescent staining in a peripheral pattern around the nucleus (Top Panel, Fig. IV 12). The intensity of this pattern of peri-nuclear staining was greater and was accompanied by the appearance of distinct punctate staining throughout the cytoplasm in cells examined 72 hrs after transfection (Bottom Panel, Fig. IV 12). The punctate staining pattern of the cytoplasm was only observed in cells that expressed intense levels of perinuclear fluorescence. The peri-nuclear and punctate staining patterns were absent in cells transfected with the pcDNA1-AMP-CAT vector (not shown).

The results of these immunofluorescence staining experiments demonstrated that *MTP3*-encoded polypeptides localized to an intracellular compartment(s) upon expression in Cos-1 cells. The pattern of fluorescence staining appeared to change as the level of *MTP3* expression increased, suggesting that over-expression of *MTP3* polypeptide may perturb its normal localization. The identity of these intracellular locations of *MTP3* polypeptide was not assessed due to the lack of appropriate antibodies for use in a double immunofluorescence staining method.

Rationale for the Prediction of the Location of a Retention or Targeting Signal in *MTP*

Results that suggested that a rat *MTP*-related protein and the recombinant *MTP3*-encoded protein expressed in Cos-1 cells are localized to intracellular membranes, possibly the Golgi, were inconsistent with the observation that the *MTP1*-encoded protein stimulated influx into *Xenopus* oocytes. However, since *MTP1* encoded a truncated protein, it was possible that it may have lacked a retention and/or targeting signal present in full length *MTP*. Several integral membrane proteins that localize to intracellular compartments contain targeting or retention signals in their amino or carboxyl terminal regions (Luzio and Banting, 1993). Since the *MTP1*-encoded protein lacked the first 83 amino acids of the amino terminus of the *MTP3*-encoded protein sequence, a retention and/or targeting signal may reside in this region. If so, the absence of the amino terminus might allow for the "escape" of *MTP1*-encoded polypeptides from the intracellular compartment to the plasma membrane.

Examination of the predicted topography of *MTP3*-encoded protein in a lipid bilayer suggested an alternate explanation for the mislocalization of *MTP1*-encoded polypeptides. Shown in Fig. IV 13 is an illustration of the proposed topography of the *MTP1*- and *MTP3*-encoded proteins, which exhibit 3 and 4 hydrophobic potential membrane-spanning regions, respectively. Of particular interest in this topographical figure are the proposed orientations of the hydrophilic amino and carboxyl termini. In the case of the *MTP3*-encoded protein, the amino and carboxyl termini would be localized to the same side of the membrane. In contrast, the amino and carboxyl termini of the *MTP1*-encoded protein would be located on opposite sides of the membrane. Thus, the failure of the truncated *MTP1*-encoded protein to be retained in the intracellular membrane compartment may simply have resulted from the carboxyl terminus being localized to the opposite side of the membrane, as compared to the carboxyl terminus of the *MTP3*-encoded protein. If the retention and/or targeting signal resided in the carboxyl terminus, its incorrect orientation may have prevented interaction with the cellular machinery responsible for retention and/or targeting.

To examine for the presence of retention or targeting signals in either termini of the *MTP* that affected its expression in the plasma membrane, amino (ΔN) and carboxyl (ΔC) terminus-deleted constructs of *MTP3* were created and expressed in *X. laevis*

oocytes. It was assumed that deletion of the retention/targeting signal would allow expression of a terminus-deleted polypeptides in the plasma membranes of oocytes, which would then give rise to increased levels of thymidine uptake. A construct of the *MTP3* cDNA that lacked the first 24 amino acids of the amino terminus was generated by the PCR method and ligated into the pcDNA1-AMP vector. This construct, termed pcDMTPAN, contained a consensus sequence for initiation of translation (5' GCCACCATGG 3', initiating ATG underlined) that was introduced as part of the PCR oligonucleotide primer. The PCR method was also used to generate a construct of *MTP3* cDNA that lacked the final 21 amino acids of the carboxyl terminus. This construct, termed pcDMTPAC, contained a translational stop codon (TAG) that was introduced as part of the PCR oligonucleotide primer. For reference, the schematic illustration of Fig. IV 14 shows the alignment of the predicted *MTP3*-, *MTP1*-, *MTPAN*- and *MTPAC*-encoded proteins and the locations of the predicted hydrophobic membrane-spanning domains.

Deletion of the Carboxyl Terminus of *MTP3* Results in Its Functional Expression at the Plasma Membrane in *X. Laevis* Oocytes

The uptake of 100 μ M [^{3} H]thymidine was examined in oocytes that had been injected with cRNA of *MTP3*, *MTP1*, *MTPAN* and *MTPAC*. As a control, thymidine uptake was also measured in oocytes that had been injected with H₂O. The results of these uptake assays are shown in Fig. IV 15. Thymidine uptake was similar for oocytes that had been injected with H₂O, *MTP3* cRNA or *MTPAN* cRNA, suggesting that the proteins encoded by the *MTP3* and *MTPAN* cDNAs did not stimulate oocyte permeability. *MTP1* cRNA-injected oocytes exhibited a 2-3 fold increase in thymidine uptake as compared to H₂O-injected oocytes, a result that was identical to that observed previously (Fig. IV 7). In contrast, oocytes that had been injected with *MTPAC* cRNA displayed an $\approx$ 13-fold increase in thymidine uptake compared to control oocytes.

These results suggested that the deletion of the carboxyl terminus of *MTP3* favored its localization to the plasma membrane of oocytes, whereupon it was capable of inducing thymidine influx. Thus, it seemed likely that the last 21 amino acids of the carboxyl terminus of *MTP* contained a retention and/or targeting signal that directed the protein to an intracellular membrane compartment.

***MTP* Appears to be a Nucleoside/Nucleotide Transporter**

The ability to obtain increased levels of thymidine uptake in *MTPAC* cRNA-injected oocytes made it possible to further characterize its' function. The experiments of Panel A of Fig. IV 16 measured the uptake of radioactivity by *MTPAC* cRNA- and H₂O-injected oocytes incubated in the presence of 100 μ M concentrations of various radiolabeled permeants. Oocytes injected with *MTPAC* cRNA exhibited a 12-fold increase in thymidine uptake and a 5-6 fold increase in uptake of uridine and adenosine relative to that of H₂O-injected oocytes. Little or no difference in sucrose uptake was observed between cRNA- and H₂O-injected oocytes, a result that differed from that obtained in similar experiments with *MTP1* cRNA-injected oocytes (Fig. IV 7). These results suggested that (i) expression of *MTPAC* induced a narrower permeant selectivity than *MTP1* and (ii) pyrimidine and purine nucleosides are permeants of *MTPAC*. Interestingly, leucine uptake was decreased $\approx$ 60% in *MTPAC* cRNA-injected oocytes compared to control oocytes.

The permeant selectivity of *MTPAC*-encoded protein was further assessed by measuring the uptake of 100 μ M [^{3} H]thymidine by *MTPAC* cRNA-injected oocytes in the absence and presence of 10 mM concentrations of non-radioactive thymidine, thymine and thymidine-5'-monophosphate (dTMP), as shown in Panel B of Fig. IV 16. The ability of 100-fold excess concentrations of thymidine or dTMP to inhibit [^{3} H]thymidine uptake suggested influx had occurred by a mediated, rather than pore-type, process. The presence of 10 mM thymine inhibited $\approx$ 60% of [^{3} H]thymidine, suggesting that the *MTPAC*-encoded transporter more readily interacted with thymidine and dTMP rather than thymine.

The demonstration that *MTPAC* cRNA-injected oocytes exhibited (i) greatly increased levels of thymidine uptake that was inhibited by structurally similar molecules, and (ii) increased levels of uptake for adenosine or uridine, but not sucrose, suggested that the *MTPAC*-encoded protein selectively mediated the flux of nucleosides, and possibly nucleotides across the plasma membrane of oocytes. Since the *MTPAC*-encoded protein lacked only the last 21 amino acids of the full length protein, it would be reasonable to assume that the permeant selectivity of this carboxyl terminus-deleted protein would reflect that exhibited by the full length protein.

Expression of Amino and Carboxyl Terminus-Deleted *MTP* in Yeast

To assess whether the amino and carboxyl terminus-deleted versions of *MTP3*-encoded protein would be functionally expressed in *S. cerevisiae*, cDNA that encompassed *MTPAN* and *MTPAC* cDNAs were cloned into the pYES2 vector to create pMTPAN and pMTPAC, respectively. These plasmids were transformed into KTK cells, which were then plated onto 2% galactose/CMM medium that contained methotrexate (50 μ g/ml), sulfanilamide (6 mg/ml), and 100 μ M thymidine, exactly as described in the experiments of Panel A, Fig. IV 6. Transformed cells failed to grow under these conditions (not shown). Soluble and membrane fractions were prepared from yeast transformed with pMTPAN, pMTPAC or pMTP1 that had been grown in 2% galactose/CMM. When these fractions were subjected to western blot analysis with the α -P and α -F antibodies exactly as described in Panel B of Fig. IV 6, immunoreactive protein was observed in the membrane fractions prepared from pMTP1-transformed cells but not from pMTPAN- or pMTPAC-transformed cells (not shown). These results suggested that the *MTPAN*- and *MTPAC*-encoded proteins, like the *MTP3*-encoded protein, were not expressed by yeast.

DISCUSSION

The isolation of the *MTP1* cDNA by expression screening for thymidine salvage in yeast, as described in Chapter III, suggested that the cDNA-encoded protein stimulated the influx of thymidine in *MTP1*-expressing yeast. In addition, the presence of several hydrophobic domains within the *MTP* sequence suggested that *MTP* was a membrane-associated protein. The objectives of the work described in this chapter were to determine if *MTP* was expressed in the plasma membrane and if *MTP*-encoded polypeptides exhibited nucleoside transport activity. Polyclonal antibodies specific for *MTP3* were produced and used to localize the full length protein or truncated derivatives

in various membrane fractions of *MTP*-expressing yeast and mammalian cells by western blot and immunofluorescence staining methods. The function of *MTP* was addressed by conducting uptake assays using *Xenopus* oocytes that had been injected with various *MTP* cRNAs.

Polyclonal antibodies specific for *MTP* were produced and characterized for use in immunological methods to determine the cellular location of *MTP*-encoded proteins. Antibodies were raised against two different types of antigens: (i) a synthetic peptide that corresponded to a hydrophilic region in the extreme amino terminus of *MTP*, and (ii) bacterial-expressed *trpE-MTP* fusion protein. The specificities of the polyclonal antibodies raised against the synthetic peptide (α -P) and the *trpE-MTP* fusion protein (α -F) were demonstrated in immunoblotting experiments. The α -P and α -F antibodies recognized immunoreactive bands that corresponded to the predicted size for the *trpE-MTP* and *malE-MTP* fusion proteins in extracts prepared from *E. coli* cells transformed with pATH11 or pMalcRI vectors that contained the same in-frame *MTP* cDNA insert. These bands were absent from extracts prepared from cells transformed with pATH11 or pMalcRI that lacked the insert. The α -P antibodies recognized an immunoreactive band in *MTP3* cDNA-transfected Cos-1 cells that exhibited the same mobility (apparent molecular weight, ≈ 23.5 kDa) in SDS-PAGE as was seen when the *MTP3* cDNA-encoded protein was produced by *in vitro* translation. The immunoreactive band identified with the α -P antibodies was nearly absent in Cos-1 cells transfected with the vector that lacked the *MTP3* cDNA. The ability of excess synthetic peptide (TFKRSRSDRFYSTRC) to prevent binding of α -P antibodies to the bacterial *malE-MTP* fusion protein or to the 23.5-kDa band in *MTP3* cDNA-transfected Cos-1 cells indicated that the α -P antibodies were specific for the peptide epitope used to raise the antibodies.

The ability of the *MTP1*-encoded protein to provide a mechanism for thymidine salvage in yeast implied that it mediated thymidine influx, which would require its presence in the plasma membrane of the yeast cells. Western blot analysis with α -F antibodies revealed the presence of immunoreactive material of ≈ 16 kDa in crude membrane fractions prepared from pMTP1-transformed KTK cells. The apparent molecular weight was similar to that observed for the *in vitro* translated product obtained from the *MTP1* cDNA (Fig. III 10, Chapter III). Immunofluorescence staining of pMTP1-transformed KTK cells with the α -F antibodies gave rise to a generalized staining over the entire cell surface. These results were consistent with the localization of *MTP1*-encoded protein to the plasma membrane of KTK cells.

Surprisingly, when KTK cells were transformed with pMTP3, which contained the full length *MTP3* cDNA, they were unable to survive under the standard selection conditions of thymidylate synthase inhibition. Immunoblotting experiments performed with α -F or α -P antibodies on extracts prepared from pMTP3-transformed cells revealed the absence of any immunoreactive material, indicating that the inability of pMTP3-transformed cells to survive thymidylate synthase inhibition was due to a lack of expression of *MTP3*-encoded protein. Since the extracts used in the immunoblotting experiments were prepared from cells that had been grown under conditions wherein expression of the cDNA-encoded protein should have been induced, it appeared that either (i) the yeast translational machinery did not recognize the translation initiation (ATG) sites, or (ii) the protein was immediately degraded upon synthesis.

As previously stated, the α -P antibodies specifically recognized an immunoreactive band in nuclei-free membrane fractions prepared from *MTP3* cDNA-transfected Cos-1 cells that was similar in molecular weight (≈ 23.5 -kDa) to that observed for the *in vitro* translated product obtained from the *MTP3* cDNA (Fig. III 10, Chapter III). The

presence of a similar ≈ 23.5 -kDa immunoreactive band in nuclei-free membrane fractions prepared from L1210/C2, S49 or HeLa cells or plasma membrane fractions of L1210/C2 cells could not be demonstrated by western blot analysis. The lack of immunoreactive material in the plasma membrane fractions of L1210/C2 cells was unexpected since the *MTP* cDNA was originally isolated from an L1210/C2 cDNA library. Although the absence of immunoreactivity could have simply been because expression of *MTP* in L1210/C2 cells occurred at very low levels, it was possible that *MTP* could have been present in a location other than the plasma membrane.

The availability of highly purified subcellular membrane fractions of rat liver cells provided an opportunity to further define the location of *MTP* provided that an *MTP*-related protein was present in rat liver cells. It was likely that a rat "homolog" of *MTP* existed since rat Walker 256 cells expressed (i) an RNA transcript that hybridized to a *MTP1* cDNA probe, and (ii) the rat RNA transcript was of similar size to the *MTP* transcript observed in L1210/C2 cells. As well, the high degree of conservation between the amino acid sequences of *MTP* and its human homolog (*HUMORF13*) suggested that antibodies raised against mouse *MTP* may cross-react with *MTP*-related proteins in other species. Immunoblotting performed with the α -P antibodies demonstrated the presence of an immunoreactive band of ≈ 23.5 kDa in the Golgi membrane fraction of rat hepatocytes. There was no evidence of similar-sized immunoreactive material in the other membrane fractions which included rough and smooth endoplasmic reticulum, mitochondria-associated membranes and mitochondria membranes. The apparent molecular weight of the α -P reactive material in Golgi membranes was the same as that exhibited by the α -P reactive material in the nuclei-free membrane fractions prepared from *MTP3* cDNA-transfected Cos-1 cells. Therefore, the ≈ 23.5 -kDa immunoreactive band present in the Golgi membrane fraction of rat liver cells likely represented a rat homolog of *MTP*.

Further support for an intracellular localization of *MTP* was provided by the patterns of immunofluorescence observed in *MTP3* cDNA-transfected Cos-1 cells. The patterns of peri-nuclear or punctate intracellular staining was dependent upon the level of expression of *MTP3*-encoded protein and was absent in cells transfected with the control vector that lacked *MTP3* cDNA. The exact locations of the *MTP*-specific staining could not be determined from the immunofluorescence studies conducted with the *MTP3* cDNA-transfected Cos-1 cells. However, the α -P antibodies specifically recognized an immunoreactive band (≈ 23.5 kDa) in the membrane, versus soluble, fractions of nuclei-free cell extracts prepared from these *MTP3* cDNA-transfected Cos-1 cells. These results suggested that the *MTP3*-encoded protein expressed in Cos-1 cells localized to the membranes of an intracellular compartment. This observation was consistent with the localization of the rat homolog of *MTP* to the Golgi membrane fraction, and suggested that *MTP* and its homologs are Golgi-specific proteins.

The expression screening method in *S. cerevisiae* was based on the premise that cells that expressed a functional mammalian NT protein in their plasma membranes would survive under conditions of inhibition of *de novo* dTMP synthesis by using exogenous thymidine. The identification of the *MTP1* cDNA by this method and the demonstration of *MTP1*-encoded protein in the yeast plasma membrane suggested that this protein may induce or mediate thymidine influx.

Xenopus laevis oocytes have been successfully used for heterologous expression of membrane proteins, including concentrative (Jarvis and Griffith, 1991; Huang *et al.*, 1993; Terasaki *et al.*, 1993; Huang *et al.*, 1994) and equilibrative mammalian nucleoside transporters (Boumah *et al.*, 1994), by injection of foreign mRNA or cRNAs. To assess the function of *MTP*-encoded proteins, uptake assays were performed using *Xenopus laevis* oocytes that had been injected with *MTP* cRNAs. Oocytes injected with *MTP1* cRNA exhibited a 2-3 fold increase in permeability to thymidine, uridine and sucrose

relative to that observed with the H₂O-injected control oocytes, whereas there were no differences in uptake of any of these substrates between *MTP3* cRNA- and H₂O-injected oocytes. Surprisingly, the injection of a carboxyl terminus-deleted *MTP* (*MTPΔC*) cRNA gave rise to a 12-13 fold increase in permeability to thymidine relative to that of H₂O-injected oocytes. The high level of functional expression observed in *MTPΔC* cRNA-injected oocytes allowed for a more thorough characterization of the induced permeability processes. The increased levels of [³H]thymidine uptake exhibited by *MTPΔC* cRNA-injected oocytes were inhibited by excess nonradioactive thymidine and dTMP, suggesting that these molecules competed as permeants for the *MTPΔC* cRNA-induced transport process. This demonstration of competition was clear evidence that the permeation process was mediated. Relative to H₂O-injected oocytes, *MTPΔC* cRNA-injected oocytes exhibited a 5-6 fold increase in uptake of adenosine and uridine and no difference in sucrose uptake, suggesting that the permeant selectivity of the induced transport process included nucleosides and nucleotides. Since the *MTPΔC*-encoded protein lacked only the carboxyl terminal 21 amino acids of the full length protein, its functional characteristics probably closely resembled those of *MTP*.

Western blot analyses revealed the presence of 16- or ~23.5-kDa immunoreactive bands in crude membrane fractions prepared from oocytes that had been injected with *MTP1* or *MTP3* cRNAs, respectively. The apparent molecular weights of these bands corresponded to those exhibited by the *in vitro* translation products of the *MTP1* and *MTP3* cDNAs. The inability of *MTP3*-encoded protein to mediate permeant influx in *X. laevis* oocytes despite its presence in membranes of oocytes was consistent with the observations that (i) the rat homolog of *MTP* was a Golgi-localized protein, and (ii) *MTP3*-encoded protein was localized to an intracellular membrane compartment upon transient expression in Cos-1 cells. Localization of the *MTP3*-encoded protein to an intracellular membrane compartment in oocytes injected with *MTP3* cRNA would therefore preclude its ability to directly mediate the flux of permeant across the plasma membrane. The altered permeability associated with the expression of the *MTP1*- and *MTPΔC*-encoded proteins in oocytes suggested that these proteins were functionally expressed at the plasma membrane. However, plasma membrane preparations from *Xenopus* oocytes are difficult to obtain and this was not directly demonstrated.

It appeared that the *MTP1*- and *MTPΔC*-encoded proteins lacked the ability to be retained in an intracellular compartment, most likely the Golgi membrane, due to the lack of a retention signal. The retention signal of *MTP* was predicted to reside within the final 21 amino acids of its carboxyl terminus since expression of a carboxyl terminus-deleted *MTP* (*MTPΔC*) in oocytes led to greatly increased levels of nucleoside uptake. In contrast, the *MTP1*-encoded protein contained the retention signal, but was likely not retained in the Golgi membrane of yeast and oocytes because of an improper orientation of its carboxyl terminus. To date, retention signals in integral membrane proteins of the Golgi compartment appear to be localized to either (i) a transmembrane domain (Luzio and Banting, 1993) or (ii) in a single case, to the amino acid sequence YQRL in the carboxyl terminus (Humphrey *et al.*, 1993). The region of the carboxyl terminus of the *MTP3*-encoded protein that appeared to be responsible for its retention in the Golgi encompassed a region of amino acid sequence (LPCTLPLKHLHSMFCQPMKTL) that contained neither a YQRL motif or a hydrophobic domain.

The studies presented in Chapters III and IV were concerned with the identification and characterization of a novel murine protein termed Mouse Transporter Protein (*MTP*). The amino acid sequence of *MTP* predicted a 25.2-kDa protein with four hydrophobic potential membrane-spanning domains. The presence of *MTP* or *MTP*-

related RNA transcripts in most mouse, rat and human cell lines and in all the mouse tissues examined, combined with the near-identity of the amino acid sequences of *MTP* and its human homolog (*HUMORF13*), suggested that *MTP* is highly conserved. *MTP* appeared to be a Golgi-localized protein, at least in hepatocytes, and the signal responsible for its retention within the Golgi may be in the carboxyl terminus. Expression of *MTP* that lacked the carboxyl terminus in *Xenopus* oocytes resulted in the appearance of a mediated transport process that appeared to accept nucleosides and nucleotides as permeants. These results suggested that *MTP* may function as a nucleoside/nucleotide transporter in the membranes of the Golgi compartment.

Interestingly, expression of *MTP* that lacked the carboxyl terminus in *Xenopus* oocytes was also observed to decrease leucine uptake relative to that observed in control oocytes. This decrease implied that *MTP* directly mediated the efflux of leucine, although other explanations are possible. For example, endogenous leucine transport in oocytes occurs primarily by a Na^+ -dependent process (Yao *et. al.*, 1994) and disruption of the plasma membrane Na^+ gradient would be expected to decrease the endogenous levels of leucine uptake. Therefore, if the *MTP1*- and *MTP Δ C*-encoded proteins disrupted the normal Na^+ gradient of the oocyte plasma membrane they could have indirectly altered leucine uptake. The exact mechanism by which *MTP* alters leucine uptake in oocytes awaits further investigation.

FIGURE IV 1

Nucleotide Sequence and Predicted Amino Acid Sequence for the Open Reading Frame of the L1210/C2 Mouse Transporter Protein (*MTP*) cDNA

The 1435-nucleotide *MTP3* cDNA sequence was determined as described in Chapter III. Shown are the assigned translational start (ATG) and the translational stop (TGA) codons of the predicted amino acid sequence for the longest open reading frame. The sequence of the synthetic peptide used to produce antibodies is indicated by the shaded region of the amino acid sequence. The amino acid sequence of *MTP1*-encoded protein is indicated by underlining.

1	AGGGCCCCGGCCAGGGCCCCAGAGACCGCTCTTTGGCTGTGGACACCGCTCTAGAGTCGTCT	67
68	GGGTTGACCCGCTGGGGCCGAAAGCTTTTGTCAAGGGGCTGGGGCAGAACGAGCCCCGGAGGTTCCG	134
135	G ATG GTG TCC ATG ACT TTC AAA CGG AGC CGC AGT GAC CGG TTC TAC AGC	183
1	Met Val Ser Met Thr Phe Lys Arg Ser Arg Ser Asp Arg Phe Tyr Ser	16
184	ACC CGG TGC TGC GGC TGT TTC CAT GTC CGC ACC GGG ACG ATC ATC CTG GGG	234
17	Thr Arg Cys Cys Gly Cys Phe His Val Arg Thr Gly Thr Ile Ile Leu Gly	33
235	ACC TGG TAC ATG GTG GTC AAC CTG TTG ATG GCC ATT TTG CTG ACC GTG GAG	285
34	Thr Trp Tyr Met Val Val Asn Leu Leu Met Ala Ile Leu Leu Thr Val Glu	50
286	GTG ACT CAT CCA AAT TCC ATG CCA GCT GTC AAC ATT CAG TAT GAA GTC ATT	336
51	Val Thr His Pro Asn Ser Met Pro Ala Val Asn Ile Gln Tyr Glu Val Ile	67
337	GGT AAT TAC TAT TCA TCT GAG AGA ATG GCT GAT AAT GCC TGC GTT CTT TTT	387
68	Gly Asn Tyr Tyr Ser Ser Glu Arg Met Ala Asp Asn Ala Cys Val Leu Phe	84
388	GCC GTC TCT GTT CTT ATG TTT ATA ATC AGT TCA ATG CTG GTT TAT GGA GCC	438
85	Ala Val Ser Val Leu Met Phe Ile Ile Ser Ser Met Leu Val Tyr Gly Ala	101
439	ATT TCT TAT CAA GTC GGC TGG CTG ATT CCT TTC TTC TGC TAC CGA CTC TTT	489
102	Ile Ser Tyr Gln Val Gly Trp Leu Ile Pro Phe Phe Cys Tyr Arg Leu Phe	118
490	GAC TTC GTT CTC AGT TGT CTG GTG GCC ATC AGT TCT CTC ACT TAC TTG CCA	540
119	Asp Phe Val Leu Ser Cys Leu Val Ala Ile Ser Ser Leu Thr Tyr Leu Pro	135
540	AGA ATC AAA GAA TAT CTG GAT CAG TTG CCT GAT TTC CCA TAC AAA GAT GAC	591
136	Arg Ile Lys Glu Tyr Leu Asp Gln Leu Pro Asp Phe Pro Tyr Lys Asp Asp	152
592	CTC CTG GCA TTG GAC TCC AGC TGC CTC CTG TTC ATC GTC CTT GTG TTC TTT	642
153	Leu Leu Ala Leu Asp Ser Ser Cys Leu Leu Phe Ile Val Leu Val Phe Phe	169
643	GTG GTA TTC ATC ATT TTT AAG GCT TAC CTA ATC AAT TGT GTT TGG GAA CCT	693
170	Val Val Phe Ile Ile Phe Lys Ala Tyr Leu Ile Asn Cys Val Trp Glu Pro	186
694	GCT ACA AAT ACA TCA ACA ACA GAA ATG TGC CAG AGA TTG CCG TGT ACC CTG	744
187	Ala Thr Asn Thr Ser Thr Thr Glu Met Cys Gln Arg Leu Pro Cys Thr Leu	203
745	CCT TTG AAA CAC CTC CAC AGT ATG TTT TGC CAA CCT ATG AAA TGG CTG TGA	795
204	Pro Leu Lys His Leu His Ser Met Phe Cys Gln Pro Met Lys Trp Leu	219
796	AGATTCTGAGAAAGAGCCACCACTCTTACCTGCGCTGCGGACATTCTGCCCTTGTCAATAAA	862
863	GCCTATACCACTTCTCTCTTTTATTTTACAGAAAGCTGCACTGCCGGGCTCTCAGACTTTGT	929
930	TGATAGAAAGTACACTGTCTTCTCTGAGCATTATTTTCAACACTAAGGAGCTTTTGGACATTTAT	996
997	TAAAACTTTTTGTTAAGTATCACATTTTAGAGCACTTTTGAAGCAATCTAGATTAAAGCAAGGCC	1063
1064	AAGTGCCATTGTTAGCCCTTAACTGAGGCTATGGGGGGAGGGGCTCTCCATGTTTTCTTTTAACT	1130
1131	TTGCACCTTCTTTGTATAAATAAGTTTATGTTTGGATGTTTGGTGGCTGAGTACITTCGGGGGC	1197
1198	GTGACTACAGTCTTGGAGATGAATAGGTTGGCTATAACACCTGCACTTTCCAGCTGATGAAACAGG	1264
1265	AGAGAGAGAGAAATACCACTTTGCACTTGCCTCTGGAAGATGATGGTTGCATGCTTCTCAATTGTACG	1331
1332	TTTCCATCTTGGTGATAAACCCCTTAAATAAGGCTCATAAATTTTAAAAA	1398
1399	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	1435

FIGURE IV 2

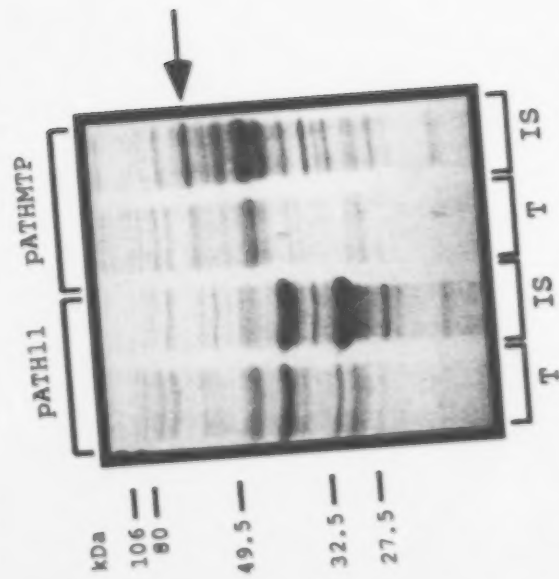
Expression of *trpE*-MTP and *malE*-MTP Fusion Proteins in *E. coli*

The ORF of the *MTP2* cDNA was cloned in-frame behind the (i) *trpE* gene in the pATH11 vector and (ii) the *malE* gene in the pMalcR1 vector to produce, respectively, the pATHMTP and pMalMTP vectors. The pATH11, pATHMTP, pMalcR1, and pMalMTP vectors were independently transformed into *E. coli* strain UT580 and expression of the non-fusion (*trpE*, *malE*) and fusion (*trpE*-MTP, *malE*-MTP) proteins was accomplished as described in Methods and Materials.

Panel A. The *trpE*-MTP fusion protein was expressed as a component of the insoluble cellular fraction in *E. coli*. The insoluble cell (IS) fraction and total cell lysate (T) were prepared from cells expressing the *trpE* (pATH11) and *trpE*-MTP (pATHMTP) fusion proteins as described in Methods and Materials. Samples (10 µg protein) were subjected to electrophoresis under reducing conditions on a 12% SDS-polyacrylamide gel. Proteins were visualized by staining with Coomassie blue. The migratory positions of BioRad Low Range Protein Standards are indicated. The arrow indicates the migratory position of the 66-kDa *trpE*-MTP fusion protein in the insoluble fraction.

Panel B. The *malE*-MTP fusion protein was expressed as a component of the insoluble cellular fraction of *E. coli*. Soluble (S) and insoluble (IS) fractions were prepared from cells that expressed the *malE* (pMalcR1) and *malE*-MTP (pMalMTP) fusion proteins as described in Methods and Materials. Samples (10 µg soluble protein, 15 µg insoluble protein) were subjected to electrophoresis under reducing conditions on a 12% SDS-polyacrylamide gel and visualized by staining with Coomassie blue. The migratory positions of Dalton Mark VII Protein Standards are indicated. The arrow indicates the migratory position of the 68 kDa *malE*-MTP fusion protein of the insoluble fraction.

A. *trpE*-MTP Fusion Protein



B. *malE*-MTP Fusion Protein

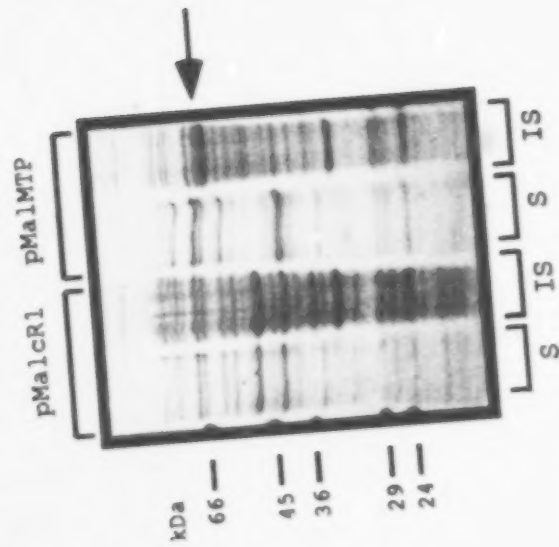


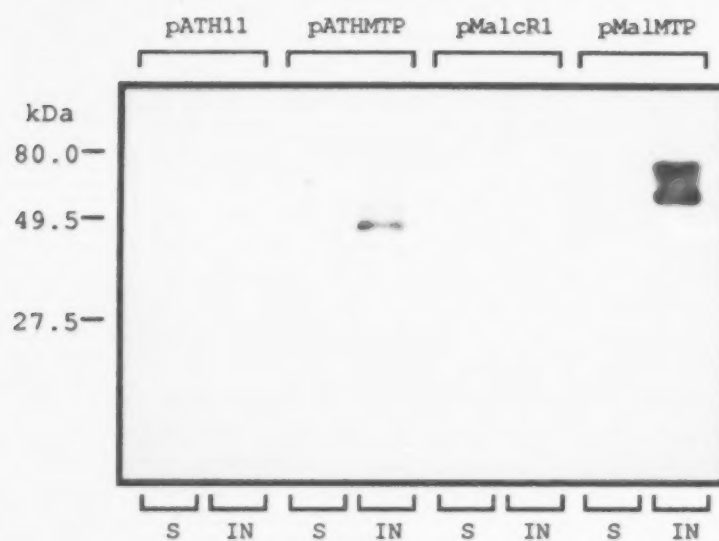
FIGURE IV 3

The Polyclonal Anti-Peptide (α -P) Antibodies Specifically Recognize *MTP*

A 15-amino acid peptide (TFKRSRSDRFYSTRC), corresponding to amino acid residues 5-19 of the predicted protein sequence of *MTP3* (Fig. IV 1), was conjugated to Diphtheria toxoid and used to produce polyclonal antibodies. The peptide (α -P) antibodies were purified by affinity chromatography on a peptide-sepharose column before being used in the immunoblotting procedure.

Panel A. The α -P antibodies recognized the *trpE-MTP* and *malE-MTP* fusion proteins. Soluble (S) and insoluble (IN) cell fractions were prepared from cells expressing non-fusion (*trpE*, *malE*) and fusion (*trpE-MTP*, *malE-MTP*) proteins. Samples that contained equivalent quantities of protein (5 μ g) were subjected to electrophoresis under reducing conditions on a 12% SDS-polyacrylamide gel and the proteins were transferred to PVDF membranes. Membranes were incubated with a 1:250 (v/v) dilution of α -P antibodies and a 1:3000 (v/v) dilution of anti-rabbit IgG-HRP conjugate, followed by detection using the enhanced chemiluminescence (ECL) method. The migratory positions of prestained BioRad Low Range Protein Standards are indicated.

Panel B. The binding of α -P antibodies to *malE-MTP* fusion protein was inhibited by free peptide. Duplicate samples (5 μ g of protein per sample) from the soluble (S) and insoluble (IN) fractions of cells expressing *malE-MTP* fusion protein were subjected to SDS-PAGE and transferred to PVDF membranes as described above. A 1:250 (v/v) dilution of α -P antibody was incubated (30 min, 4°C) in the absence or presence of 2 μ g/ml peptide (TFKRSRSDRFYSTRC) before being used in the immunoblotting procedure. The migratory positions of prestained BioRad Low Range Protein Standards are indicated.



B. Peptide Competition

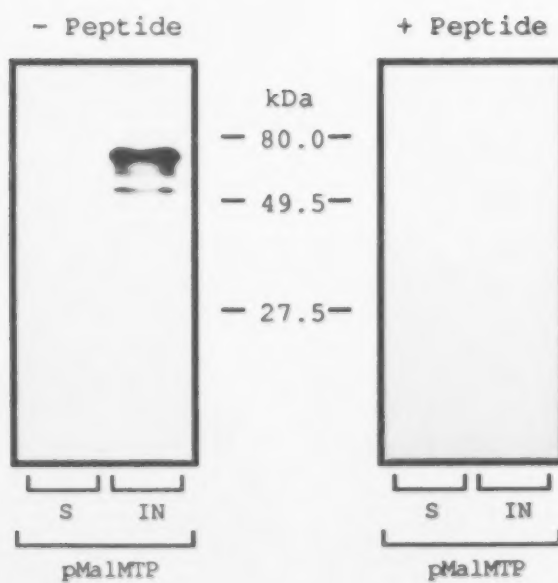


FIGURE IV 4

The Polyclonal Anti-Fusion Protein (α -F) Antibodies Specifically Recognize *MTP*

The 66-kDa *trpE-MTP* fusion protein was isolated and used as antigen to produce polyclonal antibodies as described in Methods and Materials. The fusion protein (α -F) antibodies were purified by immunoabsorption on immobilized *malE-MTP* fusion protein. Equivalent quantities (5 μ g protein) of soluble (S) and insoluble (IN) cell fractions prepared from cells expressing non-fusion (*trpE*, *malE*) and fusion (*trpE-MTP*, *malE-MTP*) proteins were electrophoresed through a 12% SDS-polyacrylamide gel and transferred to PVDF membranes. The membranes were probed with a 1:100 dilution (v/v) of α -F antibodies and a 1:3000 dilution (v/v) of anti-rabbit IgG-HRP conjugate, followed by detection using the ECL method. The migratory positions of prestained BioRad Low Range Protein Standards are indicated.

α -F Antibody

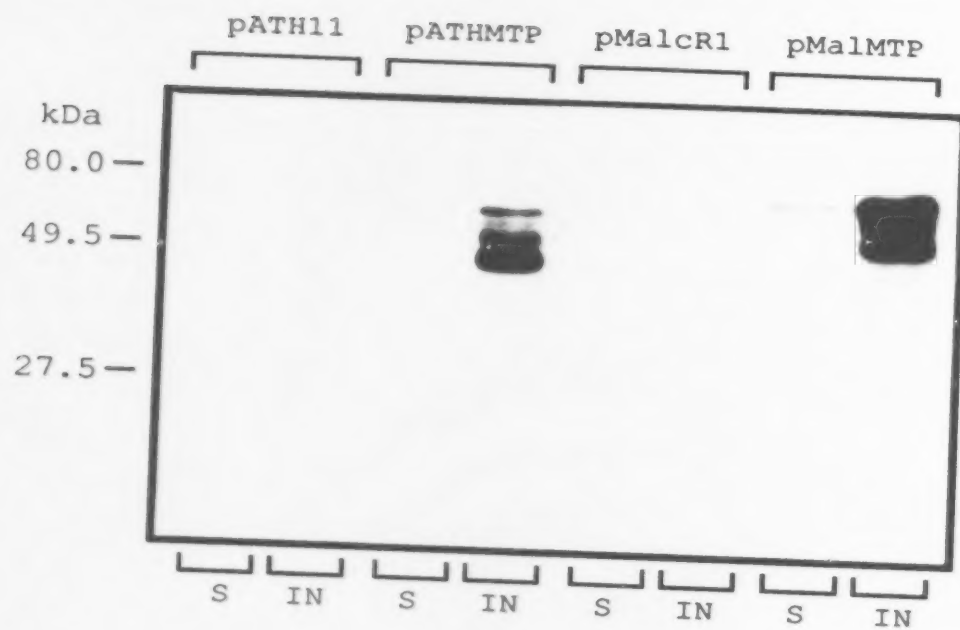


FIGURE IV 5

MTPI-Encoded Protein is Expressed in the Plasma Membrane of Yeast

KTK cells were transformed with pYES2 or pMTP1 and grown in 2% galactose/CMM that contained methotrexate (125 μ g/ml), sulfanilamide (6 mg/ml), and 1 mM or 100 μ M thymidine, respectively. Replicate portions of logarithmically growing cultures were collected by centrifugation and subjected to western blot analysis and indirect immunofluorescence staining as described in Methods and Materials.

Panel A. *MTPI*-encoded protein was expressed in the membrane fraction of pMTP1-transformed KTK cells. Soluble and crude membrane cell fractions were prepared from KTK cells transformed with pYES2 (- insert) or pMTP1 (+ insert). Samples (50 μ g protein) were subjected to electrophoresis under reducing conditions on a 12% SDS-polyacrylamide gel and the proteins were transferred to PVDF membranes. Membranes were incubated with a 1:100 dilution (v/v) of α -F antibodies and then a 1:3000 dilution (v/v) of anti-rabbit IgG-HRP conjugate. Immunoreactive protein was detected using the ECL method. The migratory positions of prestained BioRad Low Range Protein Standards are indicated.

Panel B. *MTPI*-encoded protein appeared to be expressed on the surface of pMTP1-transformed KTK cells. Sphaeroplasts were prepared from cells transformed with pYES2 (- insert) or pMTP1 (+ insert) and were immobilized on poly-L-lysine coated coverslips. The coverslips were incubated with α -F antibodies (1:100 dilution (v/v)) and rhodamine-conjugated anti-rabbit IgG (1:300 dilution (v/v)), as described in Methods and Materials. Shown are photographs of identical fields as observed under bright field or fluorescence imaging conditions. Similar results were observed in two separate immunofluorescence staining experiments.

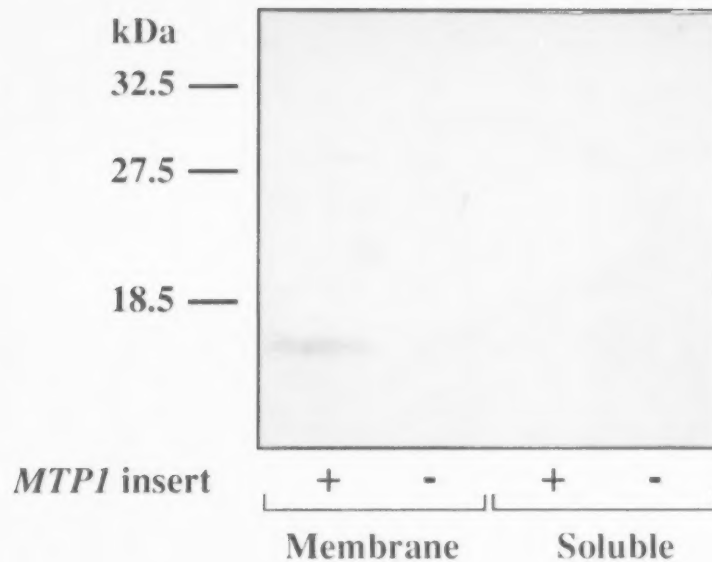
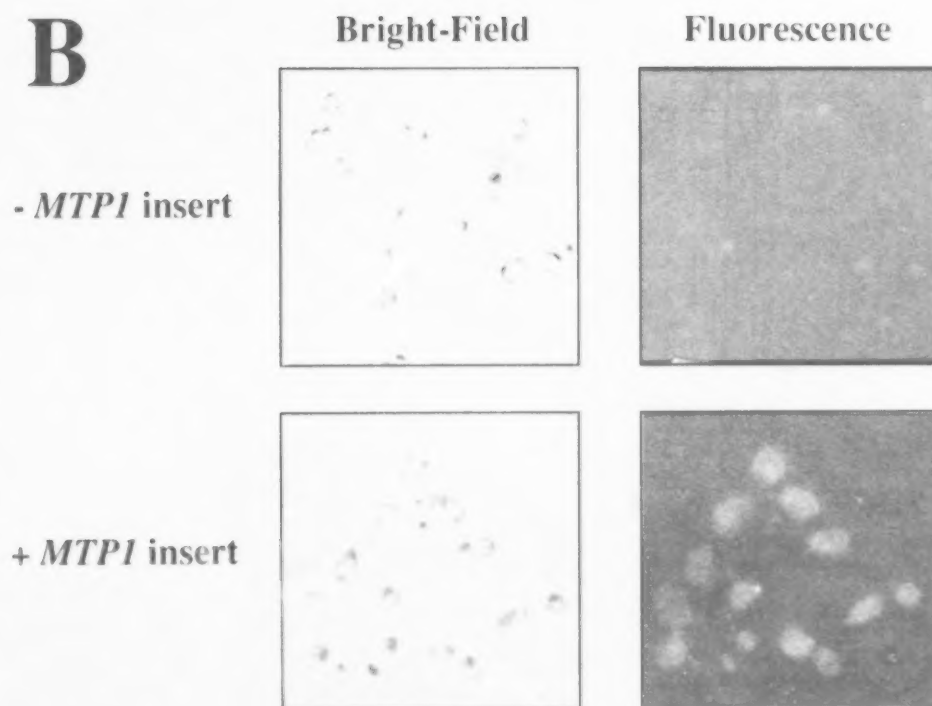
A**B**

FIGURE IV 6

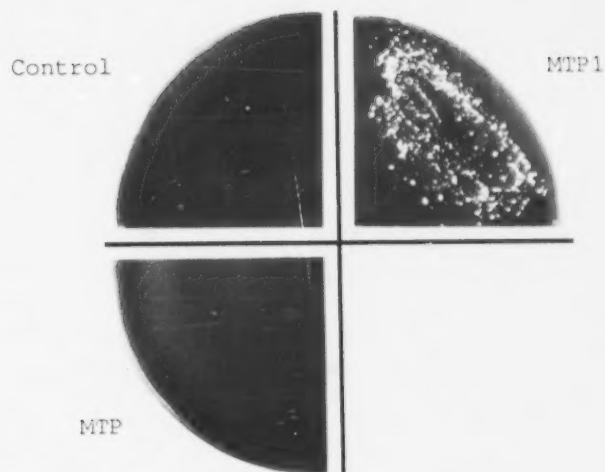
The *MTP3* cDNA-Encoded Protein Cannot be Expressed by Yeast

KTK cells were transformed with pYES2 that (i) lacked a cDNA insert (**Cont.**) (ii) contained the *MTP1* cDNA (**MTP1**), or (iii) contained the *MTP3* cDNA (**MTP**) and were grown in 2% galactose/CMM. Replicate portions of logarithmically growing cultures were then used in plating studies and western blot analysis.

Panel A. The *MTP3* cDNA was unable to confer survival of thymidylate synthase-inhibited cells in the presence of thymidine. Transformed cells were plated onto 2% galactose/CMM medium that contained methotrexate (50 $\mu\text{g/ml}$), sulfanilamide (6 mg/ml), and 100 μM thymidine. Plates were incubated at 30°C for 4 days.

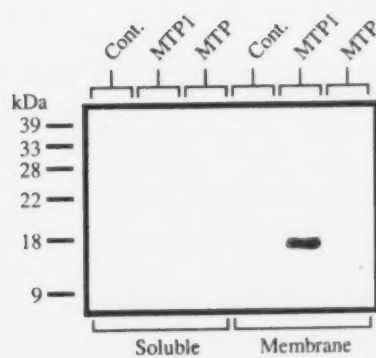
Panel B. The *MTP3*-encoded protein was not expressed by *S. cerevisiae*. Transformed cells were separated into soluble and crude membrane cell fractions by a French Press procedure. Equal quantities of fractions (25 μg protein) were subjected to 12% SDS-PAGE under reducing conditions and transferred to a PVDF membrane. The membrane was incubated with $\alpha\text{-F}$ (1:100 dilution (v/v)) or $\alpha\text{-P}$ (1:250 dilution (v/v)) and then with a 1:3000 dilution (v/v) of anti-rabbit IgG-HRP conjugate. Detection of immunoreactive proteins was performed using the ECL method. The migratory positions of the Gibco/BRL Prestained 6-60 kDa Protein Standards are indicated.

A.



B.

α -F Ab



α -P Ab

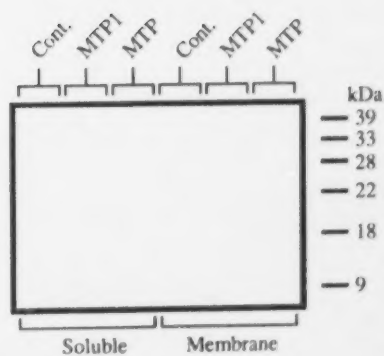


FIGURE III 7

The *MTP1*-Encoded Protein, But Not The *MTP3*-Encoded Protein, Induces Substrate Permeability Upon Expression in *Xenopus* Oocytes

The pMTP1 and pMTP3 vectors were linearized at a unique NotI restriction site and utilized as DNA template in a T7 RNA polymerase-directed transcription reaction to produce complementary RNA (cRNA). Stage VI *Xenopus laevis* oocytes were injected with either 20 nl of sterile dH₂O (Control) or cRNA solution (0.5 ng/nl) and incubated 2-3 days prior to use.

Panel A. Expression of *MTP1*-encoded protein altered substrate permeability in oocytes. Groups of oocytes (10 oocytes/assay) injected with dH₂O (Control) or *MTP1* cRNA were incubated for 30-min periods in transport buffer that contained 100 μ M [³H]thymidine (dThd), [³H]adenosine (Ado), [³H]uridine (Urd), [¹⁴C]leucine (Leu), or [¹⁴C]sucrose (Suc). Uptake was terminated by washing cells in ice-cold transport buffer and radioactivity associated with individual oocytes was measured by scintillation counting. Values presented are means $\pm$ SD (7-10 oocytes/assay).

Panel B. The *MTP1*- and *MTP3*-encoded proteins were expressed in oocytes. Crude membrane fractions were isolated from oocytes injected with dH₂O (Control), *MTP1* cRNA (MTP1), or *MTP3* cRNA (MTP), and then replicate portions of membrane fraction (25 μ g protein) were subjected to 12% SDS-polyacrylamide electrophoresis and transferred to PVDF membranes. The blots were immunostained by incubation with either α -F (1:100 dilution (v/v)) or α -P (1:250 dilution (v/v)) antibodies, followed by α -rabbit IgG-HRP conjugate (1:3000 dilution, (v/v)) and ECL reagents. The migratory positions of the Gibco/BRL Prestained 6-60 kDa Protein Ladder Standards are indicated.

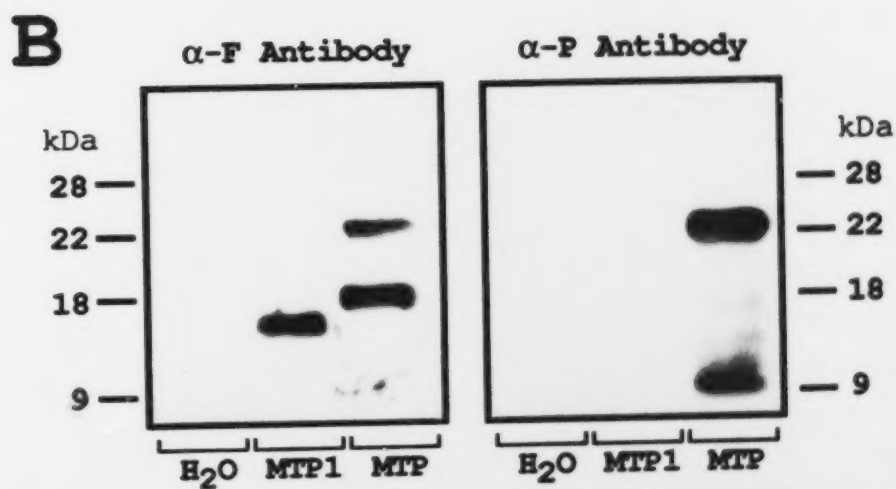
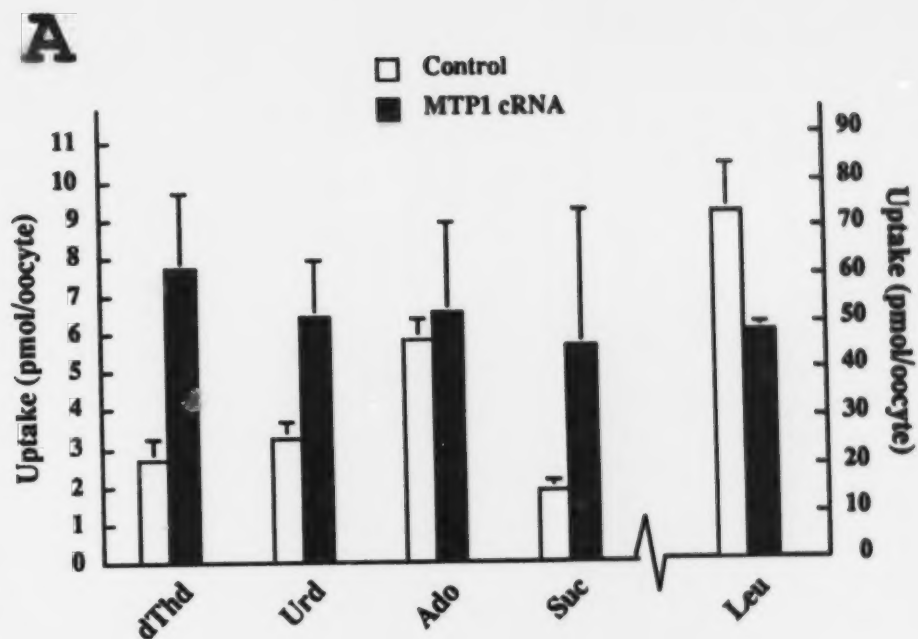


FIGURE IV 8

Characterization of *MTP3* Expression in Transiently Transfected Cos-1 Cells

Cos-1 cells were transiently transfected by the DEAE-dextran method with the pcDNA1-AMP vector that contained either the chloramphenicol transferase gene (CAT) or *MTP3* cDNA (*MTP*). Nucleus-free ("post-nuclear") cell extracts were isolated at 48 hr after transfection for use in western blot analysis.

Panel A. The ≈ 23.5 -kDa *MTP3*-encoded protein is expressed in the post-nuclear membranes of Cos-1 cells. Identical quantities (25 μ g protein) of membrane (M) and soluble (S) fractions were subjected to electrophoresis under reducing conditions on a 12% SDS-polyacrylamide gel and transferred to PVDF membranes. Membranes were subjected to the immunostaining by exposure to a 1:250 (v/v) dilution of α -P antibodies that had been preincubated (30 min, 4°C) in the absence or presence of 4 μ g/ml of peptide (TFKRSRSDRFYSTRC). The migratory positions of the BRL prestained 6-60 kDa Protein Ladder Standards are indicated.

Panel B. Migration of the *MTP3*-encoded protein is constant under different conditions of SDS-PAGE. Replicate samples (25 μ g protein) of post-nuclear membranes were solubilized in loading buffer in the presence (reduced: +) or absence (reduced: -) of β -mercaptoethanol. Samples were then either loaded directly on a 12% SDS-polyacrylamide gel or heated at 90°C for 5 min prior to gel loading. After electrophoresis and transfer to PVDF membranes, blots were immunostained with a 1:250 (v/v) dilution of α -P antibody. The migratory positions of the BRL prestained 6-60 kDa Protein Ladder Standards are indicated.

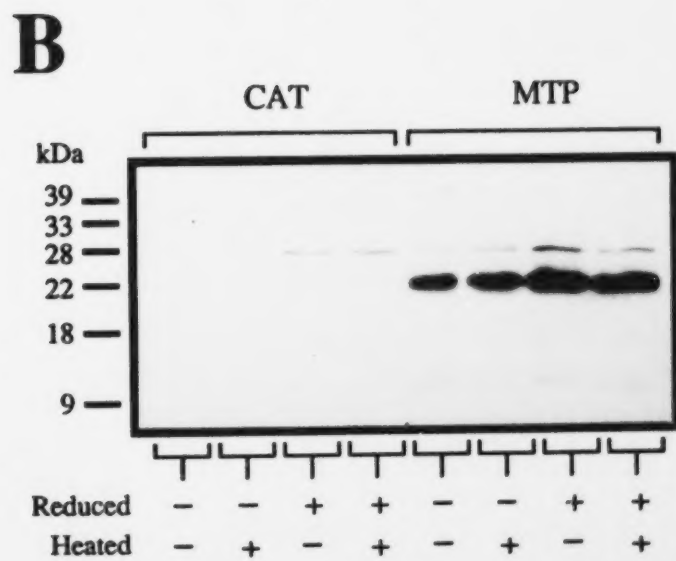
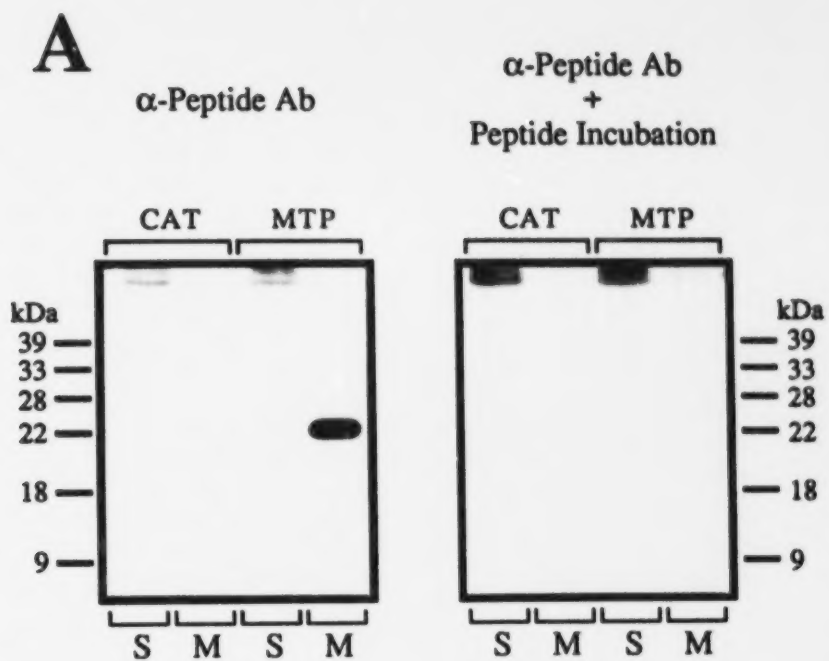


FIGURE IV 9

Normal Levels of *MTP* Expression Are Low in Cultured Cells

Post-nuclear membranes were isolated from actively proliferating cultured L1210/C2, S49, HeLa, and Cos-1 cells and equivalent portions (25 μ g protein) of reduced membrane samples were subjected to electrophoresis on a 12% SDS-polyacrylamide gel. For comparative purposes, a sample (5 μ g protein) of post-nuclear membranes prepared from Cos-1 cells transiently transfected with pcDMTP3 was also included. The electrophoretically separated samples were transferred to a PVDF membrane and the membrane was immunostained with a 1:250 (v/v) dilution of α -P antibodies. For reference, the migratory positions of the Gibco/BRL Prestained 6-60 kDa Protein Ladder Standards are indicated.

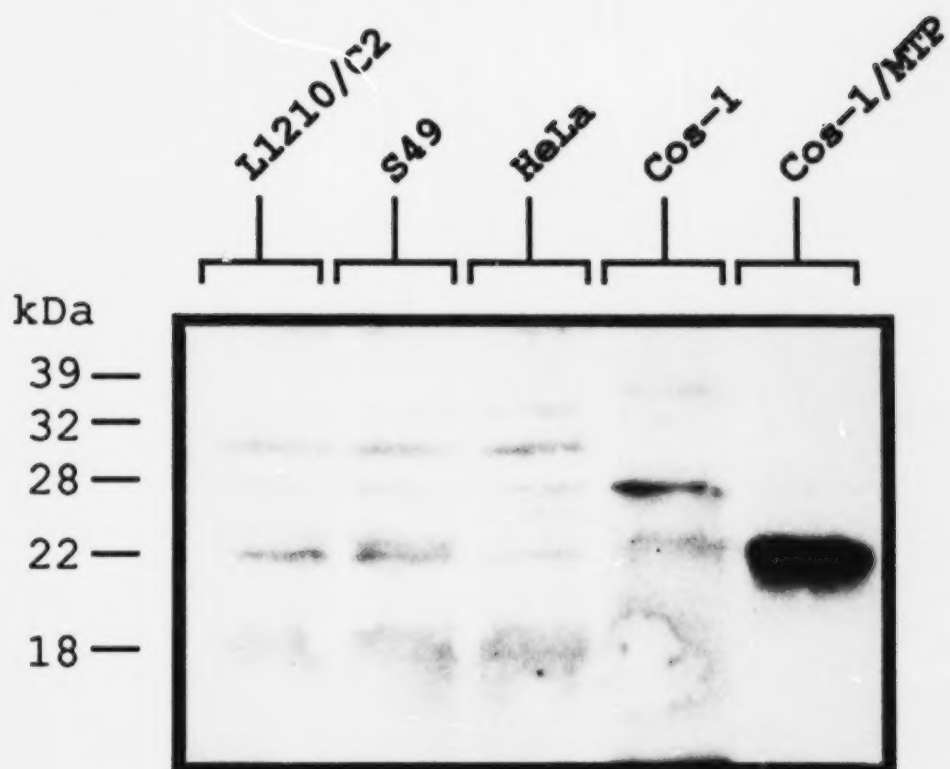


FIGURE IV 10

A Protein Related to *MTP* is Localized to the Golgi-Enriched Membrane Fraction of Rat Liver Cells

Subcellular membrane fractions of rat liver cells were isolated in the laboratory of J. Vance (University of Alberta) and samples that contained equal quantities of protein (25 μ g) were solubilized under reducing conditions and subjected to electrophoresis on a 12% SDS-polyacrylamide gel prior to transfer to a PVDF membrane. The membrane was sequentially incubated with α -F (1:100 dilution (v/v)) antibodies and anti-rabbit IgG-HRP conjugate (1:3000 dilution (v/v)) and then the immunoreactive proteins were detected by the ECL method. Abbreviations are: **Total**, total cellular protein; **Golgi**, Golgi-enriched fraction; **Smooth-ER**, smooth endoplasmic reticulum-enriched fraction; **Rough-ER**, rough endoplasmic reticulum-enriched fraction; **C-Mito**, crude mitochondria fraction; **P-Mito**, purified mitochondria fraction; **M A M**, mitochondria-associated membrane fraction. The migratory positions of the Gibco/BRL Prestained 6-60 kDa Protein Ladder Standards are indicated.

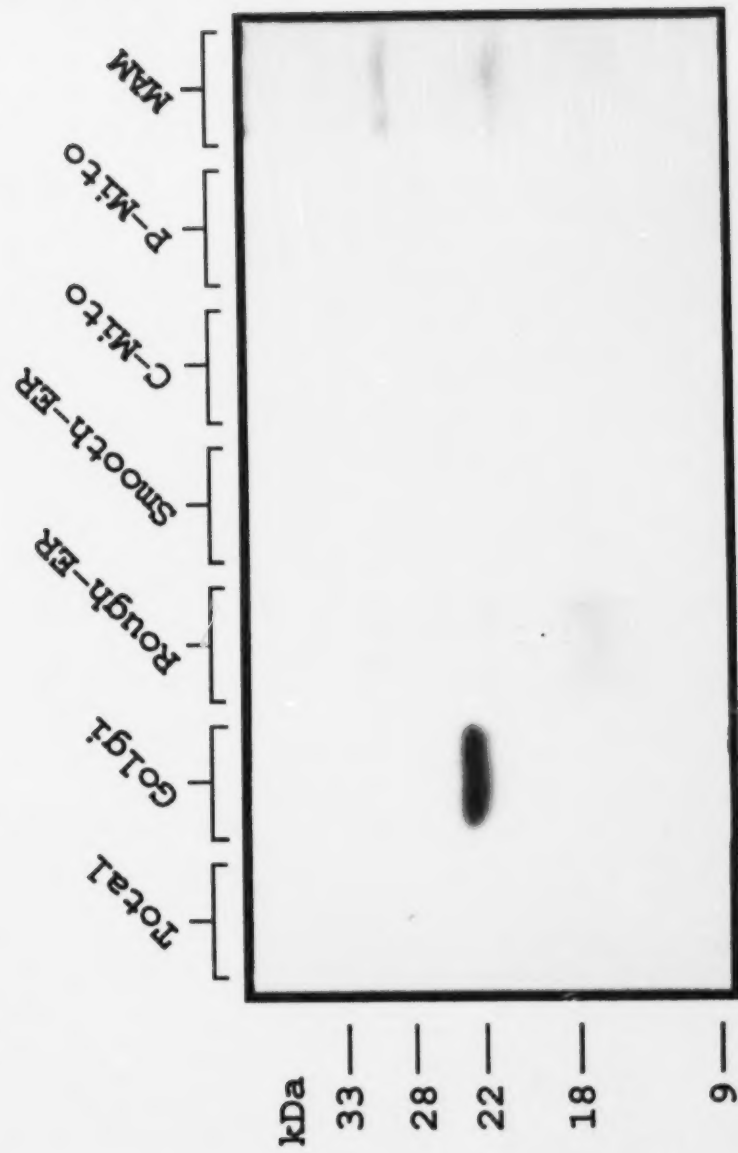


FIGURE IV 11

Immunofluorescence Staining Demonstrates *MTP3* to be Localized in an Intracellular Region Upon Expression in Cos-1 Cells

Cos-1 cells were transiently transfected by the DEAE-dextran method with pcDNA1-AMP vector that contained the CAT gene (pcDNA1-AMP-CAT) or *MTP3* cDNA (pcDMTP3) and allowed to grow for 18 hr. Transfected cells were transferred to glass coverslips and allowed to attach for 4 hr, whereupon cells were subjected to indirect immunofluorescence staining as described in Methods and Materials. Briefly, cells were fixed with paraformaldehyde, permeabilized, and incubated with α -P antibodies (1:50 dilution (v/v)). Following a second incubation with α -rabbit IgG-rhodamine conjugate (1:300 dilution (v/v)), coverslips were mounted in glycerol-gelatin and visualized by fluorescence microscopy. Shown are photographs of the bright field and fluorescent images of three different fields (40x magnification) from a culture of cells transfected with the pcDMTP3 vector. No appreciable level of fluorescent staining was present in cultures of Cos-1 cells transfected with the pcDNA1-AMP-CAT vector, therefore photographs of these cells are not shown.

Bright-Field

Fluorescence

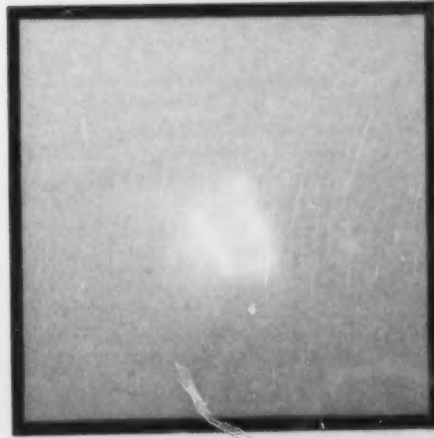
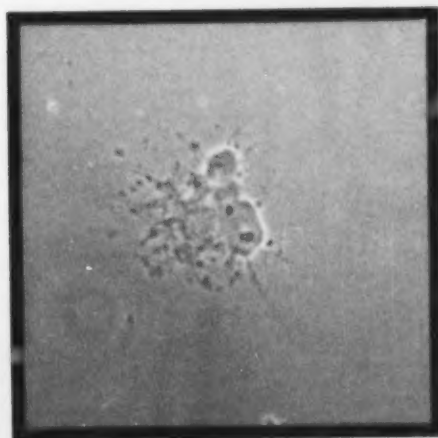
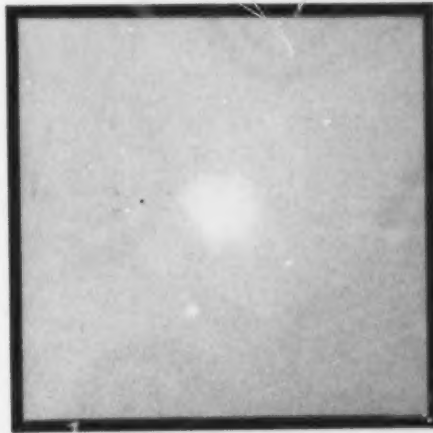
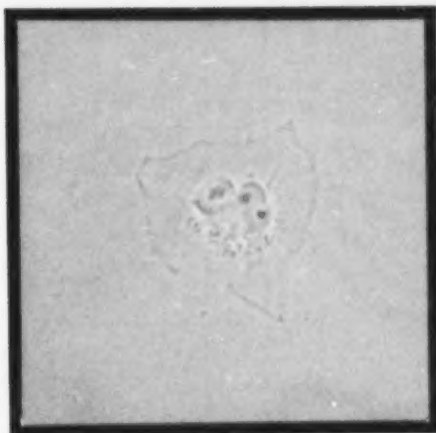
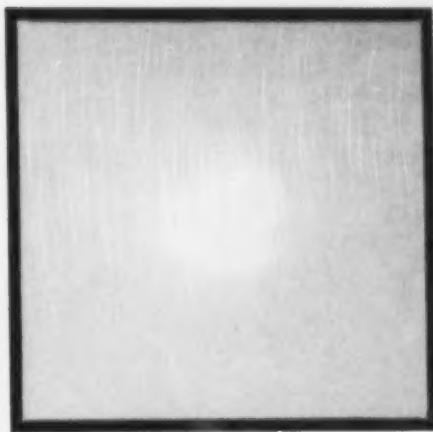
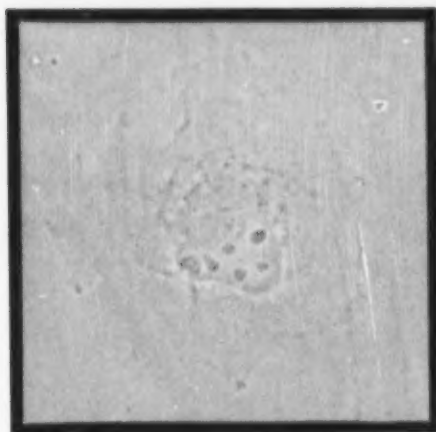
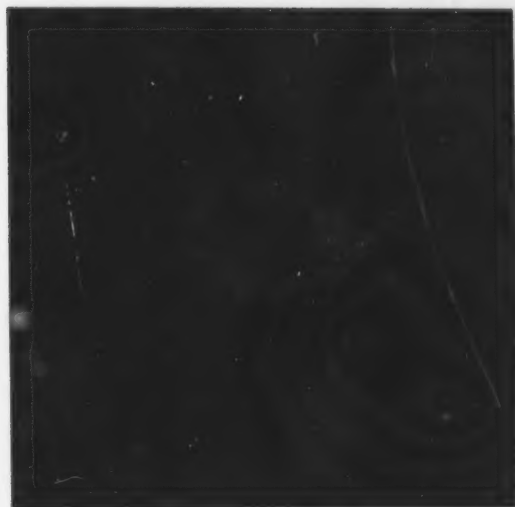


FIGURE IV 12

Transiently-Expressed *MTP3* Appears to be Localized in a Peri-Nuclear Region in Cos-1 Cells

Cos-1 cells were transiently transfected with the pcDMTP3 vector as described in Fig. IV 11 and transferred to coverslips 24 hr after the transfection event. Cell cultures were subjected to the immunofluorescence staining with the α -P antibodies (1:50 dilution (v/v)) at 48 (Top Panel) and 72 hr (Bottom Panel) after transfection. Following a second incubation with anti-rabbit IgG-rhodamine conjugate (1:300 dilution (v/v)), coverslips were mounted in glycerol-gelatin and visualized by fluorescence microscopy. Shown are the photographs of fluorescent images (63x magnification).

48 Hours



72 Hours

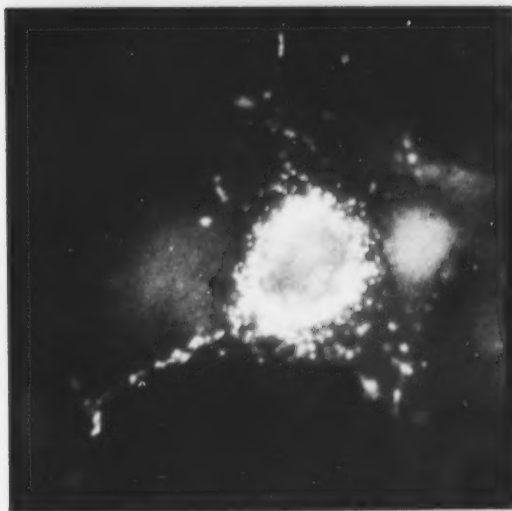


FIGURE IV 13

Proposed Topographical Orientation of *MTP1* and *MTP3*

Shown is an illustration of the proposed topography of *MTP1* and *MTP3* in a lipid bilayer based upon the hydropathic profile predicted by the Kyte-Doolittle Algorithm. The lipid bilayer is indicated by the lightly-shaded horizontal band, the hydrophobic membrane-spanning domains are shown as vertical tubes, and the amino and carboxy termini are designated by N and C, respectively.

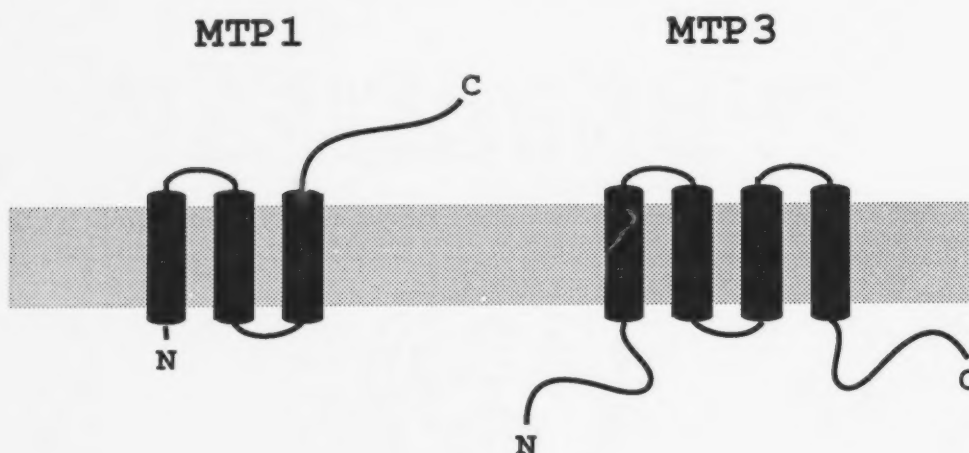


FIGURE IV 14

Schematic Illustration of the *MTP* cDNA Constructs

Shown are schematic representations of the proteins encoded by the *MTP3*, *MTP1*, *MTP Δ N* and *MTP Δ C* cDNAs. The hydropathic profile of the full length *MTP3*-encoded protein and the orientation of the amino (N) and carboxyl (C) termini are indicated at the top of the figure. Proteins are represented by elongated boxes drawn on the same scale as the hydropathic profile and the predicted hydrophobic membrane-spanning regions are indicated by the shaded boxes. The construction of the *MTP Δ N* and *MTP Δ C* cDNAs are described in Methods and Materials. *MTP1* and *MTP Δ N* lacked the first 83 and 24 amino acids, respectively, of the *MTP3*-encoded protein. *MTP Δ C* lacked the final 21 amino acid residues of the carboxyl terminus.

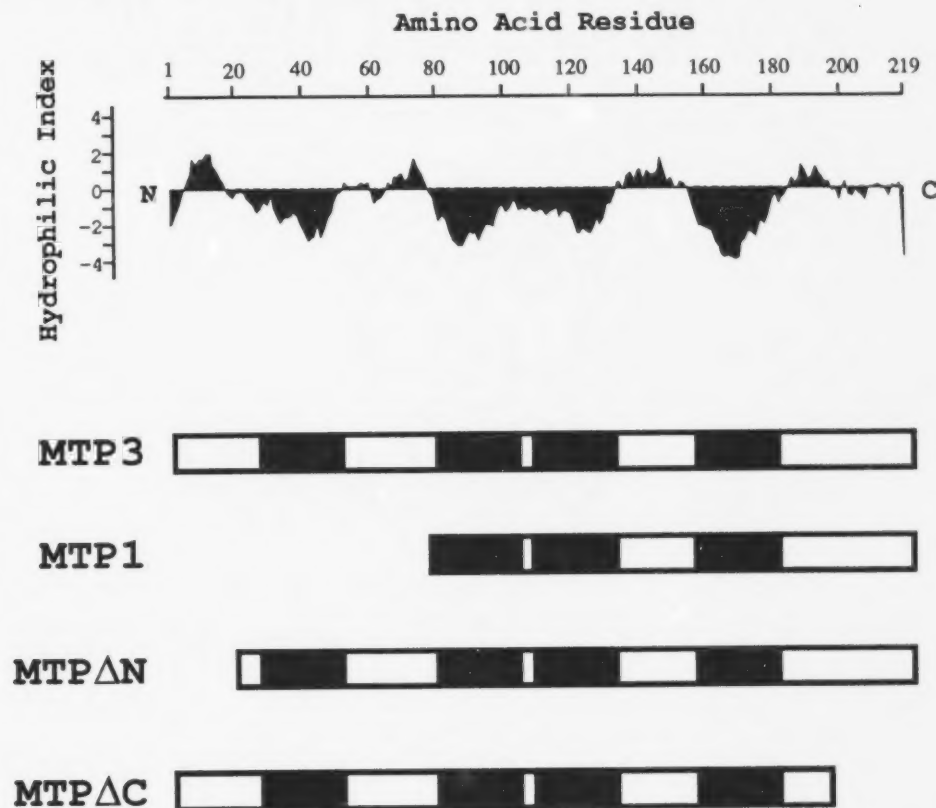


FIGURE IV 15

Thymidine Permeability is Greatly Increased Upon Expression of *MTPΔC* in *Xenopus* Oocytes

The pcDNA1-AMP vectors that contained the *MTP3*, *MTP1*, *MTPΔN* and *MTPΔC* cDNAs were linearized and used as template to produce their respective cRNAs as described in Methods and Materials. *X. laevis* oocytes (10 oocytes per condition) were injected with either 20 nl of sterile dH₂O (Control) or cRNA solution (0.5 ng/nl) and incubated 3 days prior to use in uptake assays, as described in Fig. IV 7. Uptake assays were conducted over 30-min periods in transport buffer that contained 100 μM [³H]thymidine. Values presented are means ± SD (6-9 oocytes/assay).

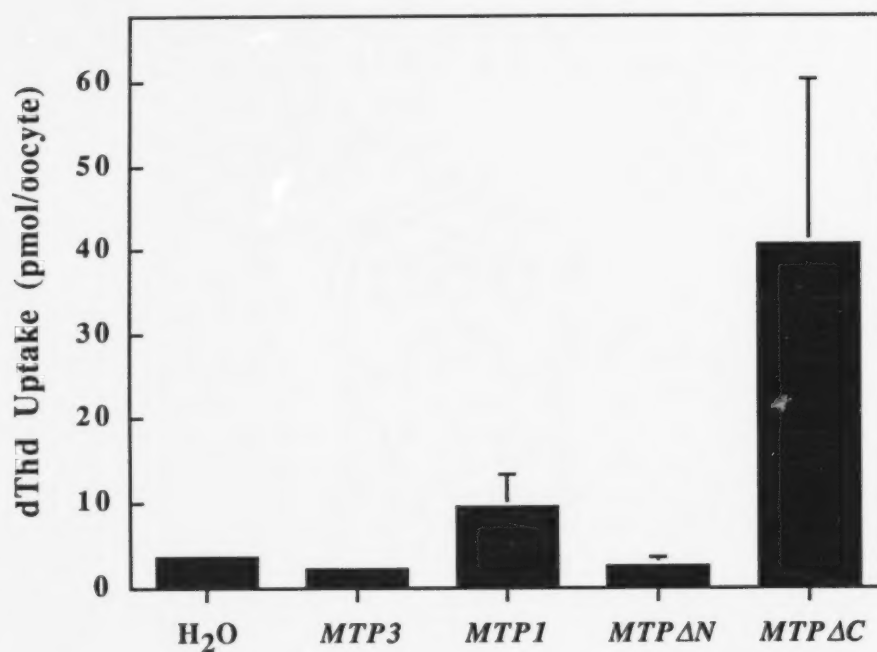


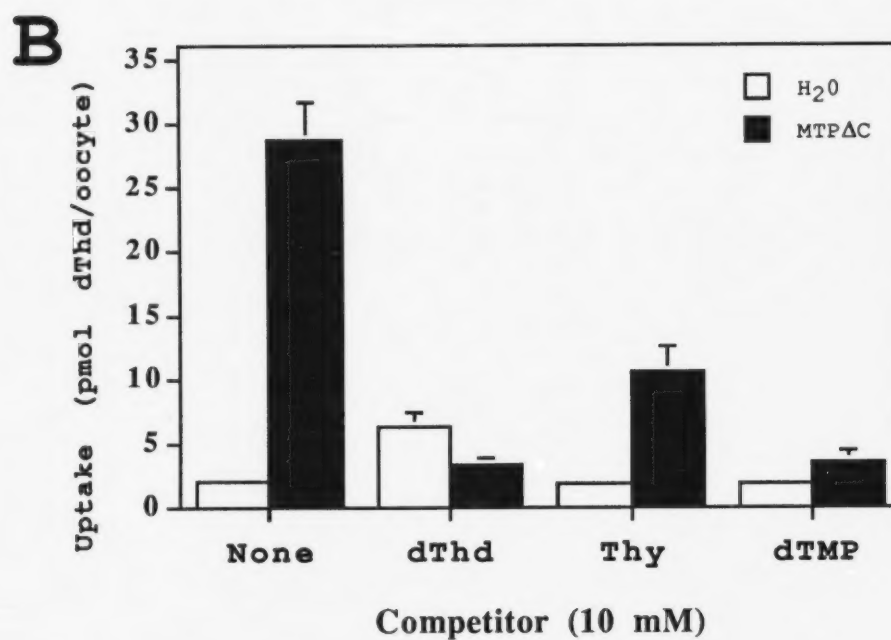
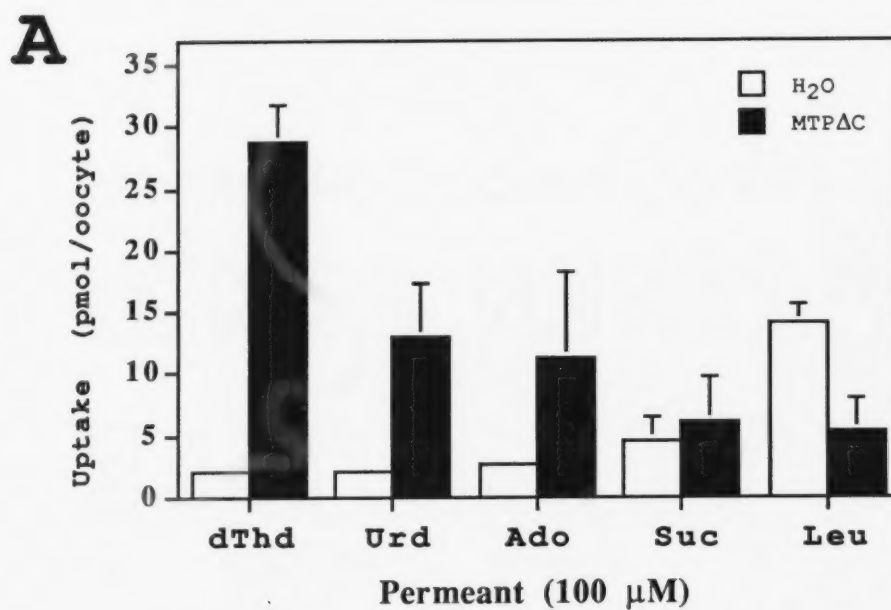
FIGURE IV 16

***MTPΔC* Accepts Nucleosides and Nucleotides as Permeants Upon Expression in *Xenopus* Oocytes**

MTPΔC cRNA was synthesized as described in Methods and Materials and *X. laevis* oocytes (10 oocytes per condition) were injected with either 20 nl of sterile dH₂O (Control) or cRNA solution (0.5 ng/nl) and incubated 3 days prior to use in uptake assays

Panel A. Expression of *MTPΔC*-encoded protein induces a selective uptake of organic solutes. Groups of oocytes (10 oocytes/assay) injected with dH₂O (Control) or *MTPΔC* cRNA were incubated for 30 min periods in transport buffer that contained 100 μM concentrations of [³H]thymidine (**dThd**), [³H]adenosine (**Ado**), [³H]uridine (**Urd**), [¹⁴C]leucine (**Leu**), or [¹⁴C]sucrose (**Suc**). Uptake was terminated by washing cells in ice-cold transport buffer and radioactivity associated with individual oocytes was measured by scintillation counting. Values presented are means ± SD (7-10 oocytes/assay).

Panel B. Thymidine uptake mediated by *MTPΔC*-encoded protein is inhibited in the presence of dTMP. Groups of oocytes (10 oocytes/assay) injected with dH₂O (Control) or *MTPΔC* cRNA were incubated for 30-min periods in transport buffer that contained 100 μM [³H]thymidine alone or with 10 mM non-radioactive thymidine (**dThd**), thymine (**Thy**), or thymidine-5'-monophosphate (**dTMP**). Oocytes were processed as described above and values presented are means ± SD (6-8 oocytes/assay).



BIBLIOGRAPHY

- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1989) Current protocols in molecular biology.
- Barnard, E. A. (1992) Receptor classes and the transmitter-gated ion channels. *Trends Biochem. Sci.* **17**, 368-374.
- Boumah, C. E., Harvey, C. M., Paterson, A. R. P., Baldwin, S. A., Young, J. D., and Cass, C. E. (1994) Functional expression of the nitrobenzylthioinosine-sensitive nucleoside transporter of human choriocarcinoma (BeWo) cells in isolated oocytes of *Xenopus laevis*. *Biochem. J.* **299**, 769-773.
- Capasso, J. M., Keenan, T. W., Abeijon, C., and Hirschberg, C. B. (1989) Mechanism of phosphorylation in the lumen of the Golgi apparatus. *J. Biol. Chem.* **264**, 5233-5240.
- Chambers, S. P., Prior, S. E., Barstow, D. A., and Minton, N. P. (1988) The pMTL *nic*-cloning vectors. I. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. *Gene* **68**, 139-149.
- Croze, E. M. and Morre, D. J. (1984) Isolation of plasma membrane, Golgi apparatus and endoplasmic reticulum fractions from single homogenates of mouse liver. *J. Cell. Physiol.* **119**, 46-57.
- Gunekunst, K. A., Rao, G. N., and Church, R. L. (1990) Molecular cloning and complete nucleotide sequence of the cDNA encoding a bovine lens intrinsic membrane protein (MP19). *Curr. Eye Res.* **9**, 955-961.
- Harlow, E. and Lane, D. (1988) Antibodies - a laboratory manual. Cold Spring Harbor Laboratory.
- Hirschberg, C. B. and Snider, M. D. (1987) Topography of glycosylation in the rough endoplasmic reticulum and Golgi apparatus. *Ann. Rev. Biochem.* **56**, 63-87.
- Hogue, D. L., Hodgson, K. C., and Cass, C. E. (1990) Effects of inhibition of N-linked glycosylation by tunicamycin on nucleoside transport polypeptides of L1210 leukemia cells. *Biochem. Cell Biol.* **68**, 199-209.
- Huang, Q., Harvey, C. M., Paterson, A. R. P., Cass, C. E., and Young, J. D. (1993) Functional expression of Na⁺-dependent nucleoside transport systems of rat intestine in isolated oocytes of *Xenopus laevis*. *J. Biol. Chem.* **268**, 20613-20619.
- Huang, Q. Q., Yao, S. Y. M., Ritzel, M. W. L., Paterson, A. R. P., Cass, C. E., and Young J. D. (1994) Cloning and functional expression of a complementary DNA encoding a mammalian nucleoside transporter protein. Submitted for Publication.
- Humphrey, J. S., Peters, P. J., Yuan, L. C., and Bonifacino, J. S. (1993) Localization of TGN38 to the *trans*-Golgi network: involvement of a cytoplasmic tyrosine-containing sequence. *J. Cell Biol.* **120**, 1123-1135.
- Jarvis, S. M. and Griffith, D. A. (1991) Expression of the rabbit intestinal N2 Na⁺/nucleoside transporter in *Xenopus laevis* oocytes. *Biochem. J.* **278**, 605-607.

- Kaldi, K., Diestelkotter, P., Stenbeck, G., Auerbach, S., Jakle, U., Magert, H. J., Wieland, F. T., and Just, W. W. (1993) Membrane topology of the 22 kDa integral peroxisomal membrane protein. *Fed. Eur. Biochem. Soc.* **315**, 217-222.
- Koerner, T.J., Hill, J.E., Myers, A.M., and Tzagoloff, A. (1991) High-expression vectors with multiple cloning sites for construction of *trpE* fusion genes: pATH vectors. *Meth. Enzymol.* **194**, 477-490.
- Kozak, M. (1991) An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell Biol.* **115**, 887-903.
- Kumar, N. M., Jarvis, L. J., Tenbroek, E., and Louis, C. F. (1993) Cloning and expression of a major rat lens membrane protein, MP20. *Exp. Eye Res.* **56**, 35-43.
- Kyte, J. and Doolittle, R. F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105-132.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of bacteriophage T4. *Nature* **227**, 680-685.
- Lin, W. and Culp, L. A. (1991) Selectable plasmid vectors with alternate and ultrasensitive histochemical marker genes. *BioTechniques* **11**, 344-351.
- Luzio, P. J. and Banting, G. (1993) Eukaryotic membrane traffic: retrieval and retention mechanisms to achieve organelle residence. *Trends Biochem. Sci.* **18**, 395-398.
- Maina, C.V., Riggs, P.D., Grandea, A.G., Slatko, B.E., Moran, L.S., Tagliamonte J.A., McReynolds, L.A., and Guan, C. (1988) An *Escherichia coli* vector to express and purify foreign proteins by fusion to and separation from maltose-binding protein. *Gene* **74**, 365-373.
- Milla, M. E., Clairmont, C. A., and Hirschberg, C. B. (1992) Reconstitution into proteoliposomes and partial purification of the Golgi apparatus membrane UDP-galactose, UDP-xylose, and UDP-glucuronic acid transport activities. *J. Biol. Chem.* **267**, 103-107.
- Milla, M. E. and Hirschberg, C. B. (1987) Reconstitution of Golgi vesicle CMP-sialic acid and adenosine-3'-phosphate-5'-phosphosulfate transport into proteoliposomes. *Proc. Natl. Acad. Sci. USA* **86**, 1786-1790.
- Mitra, R. S. and Bernstein, I. A. (1970) Thymidine incorporation into deoxyribonucleic acid by isolated rat liver mitochondria. *J. Biol. Chem.* **245**, 1255-1260.
- Momura, N., Miyajima, N., Kawarabayashi, Y., and Tabata, S. (1993) Prediction of new human genes by entire sequencing of randomly sampled cDNA clones. Unpublished Kazusa DNA Research Institute, Japan.
- Morimyo, M., Hongo, E., Hama-Inaba, H., and Machida, I. (1992) Cloning and characterization of the *mvrC* gene of *Escherichia coli* K-12 which confers resistance against methyl viologen toxicity. *Nuc. Acids Res.* **20**, 3159-3165.
- Pisoni, R. L. and Thoene, J. G. (1989) Detection and characterization of a nucleoside transport system in human fibroblast lysosomes. *J. Biol. Chem.* **264**, 4850-4856.

- Purewal, A. S. (1991) Nucleotide sequence of the ethidium efflux gene from *Escherichia coli*. *Fed. Eur. Microbiological. Soc. Microbiol. Letters* **82**, 229-232.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular cloning - a laboratory manual. Cold Spring Harbour Laboratory.
- Sasatsu, M., Shibata, Y., Noguchi, N., and Kono, M. (1992) High-level resistance to ethidium bromide and antiseptics in *Staphylococcus aureus*. *Fed. Eur. Microbiological. Soc. Microbiol. Letters* **93**, 109-114.
- Scotland, P. B., Colledge, M., Melnikova, I., Dai, Z., and Froehner, S. C. (1993) Clustering of the acetylcholine receptor by the 43-kD protein: involvement of the zinc finger domain. *J. Cell Biol.* **123**, 719-738.
- Sigel, E. (1990) Use of *Xenopus* oocytes for the functional expression of plasma membrane proteins. *J. Memb. Biol.* **117**, 201-221.
- Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A. K., Gartner, F.H., Provenzano, M.D., Fujimoto, E. K., Goeke, N.M., Olsen, B.J., and Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76 - 85.
- Terasaki, T., Kadowaki, A., Higashida, H., Nakayama, K., Tamai, I., and Tsuji, A. (1993) Expression of the Na⁺-dependent nucleoside transport system in rabbit small intestine: studies with mRNA-injected *Xenopus laevis* oocytes. *Biol. Pharm. Bull.* **16**, 493-496.
- Vance, J. E. (1990) Phospholipid synthesis in a membrane fraction associated with mitochondria. *J. Biol. Chem.* **265**, 7248-7256.
- Vance, J. E. and Vance, D. E. (1988) Does rat liver Golgi have the capacity to synthesize phospholipids for lipoprotein secretion? *J. Biol. Chem.* **263**, 5898-5909.
- Walker, L. F. and Lewis, R. A. (1987) The metabolism of deoxyguanosine in mitochondria. Characterization of the uptake process. *Mol. Cell. Biochem.* **77**, 71-77.
- Weiher, H., Noda, T., Gray, D. A., Sharpe, A. H., and Jaenisch, R. (1990) Transgenic mouse model of kidney disease: insertional inactivation of ubiquitously expressed gene leads to nephrotic syndrome. *Cell* **62**, 425-434.
- Yao, S. Y. M., Muzyka, W. R., Elliot, J. F., Cheeseman, C. I., and Young, J. D. (1994) PolyA⁺ RNA from the mucosa of rat jejunum induces novel Na⁺-dependent and Na⁺-independent leucine transport activities in oocytes of *Xenopus laevis*. *Mol. Memb. Biol.* **In Press**.

Chapter V

***Spodoptera frugiperda* (Sf9) CELLS EXPRESS NOVEL PURINE- AND PYRIMIDINE-SELECTIVE NUCLEOSIDE TRANSPORTERS¹**

¹ A version of this chapter has been accepted for publication: Hogue, D. L. and Cass, C. E. (1994) *Insect Biochem. Mol. Biol.*

INTRODUCTION

This study encompasses a characterization of the NT processes present in the cultured *Spodoptera frugiperda* ovary (Sf9) cell line, a lepidopteran cell type that has been used extensively for the over-expression of mammalian proteins via the use of baculovirus expression vectors. Recombinant proteins are generally correctly translated, modified, and targeted within Sf9 cells, and in most cases, are functionally and antigenically similar to their authentic mammalian counterparts (Luckow and Summers, 1988; Maeda, 1989). The NT processes of Sf9 cells were examined to ascertain the potential usefulness of this cell type for the functional expression and characterization of a recombinant mammalian NT protein, a situation in which Sf9 cells would (i) necessarily lack endogenous NT proteins that carry out the same role or (ii) express NT processes that are readily discernable from that mediated by the recombinant NT protein.

Processes that mediate the transport of nucleosides into non-mammalian eucaryotic cell types have not been well characterized with respect to permeant selectivity, kinetic parameters of transport, or sensitivity to NT inhibitors. What little data are available suggest that significant differences exist between NT processes of non-mammalian and mammalian cell types.

The yeast *Saccharomyces cerevisiae* exhibits transport processes selective for only cytidine or uridine and lacks the capacity for purine nucleoside and thymidine transport (Grenson, 1969). Grenson (1969) demonstrated that cytidine and cytosine share a common transport process and uridine enters *S. cerevisiae* by a shared process with uracil and also by a second uridine-specific process. While the genes encoding the cytosine and uracil permeases of *S. cerevisiae* have recently been identified and isolated (Weber *et al.*, 1990; Jund *et al.*, 1988), it is not known if these permeases accept either cytidine or uridine as permeants. In *Candida albicans*, pyrimidine nucleosides and thymidine have been shown to share a common high-affinity transport process (K_m values: 80-400 μ M) and uridine transport in this yeast is mediated by a uridine-specific process (K_m value: 40 μ M) similar to that observed in *S. cerevisiae* (Rao *et al.*, 1983). Transport of uridine and adenosine into *Schizosaccharomyces pombe* by an energy-dependent process has been reported (Foury and Goffeau, 1975), although initial rates of transport and permeant selectivity of the process(es) responsible were not determined.

Mosquito *Aedes albopictus* cells express a high-affinity NT process that accepts both purine and pyrimidine nucleosides (K_m values: low micromolar range) and exhibits low sensitivity to inhibition by NBMPR (Sherwood and Stollar, 1982; Abidi *et al.*, 1987). This NT process is sensitive to inhibition by low micromolar concentrations of dipyrindamole. Interestingly, erythrocytes of the primitive vertebrate *Epartretus stouti* (hagfish) possess a similar high-affinity NT process that is NBMPR-insensitive and dipyrindamole-sensitive (Fincham *et al.*, 1991). It appears that these mosquito and hagfish NT processes exhibit a degree of similarity to the mammalian *ei* NT processes.

Parasitic protozoa, intraerythrocytic parasites, and helminths generally lack pathways for *de novo* purine and/or pyrimidine synthesis and depend upon salvage of preformed nucleobases and nucleosides for metabolic requirements. These eucaryotes have been shown to express high-affinity NT processes that exhibit a permeant-selectivity and inhibitor-insensitivity not observed in mammalian cells (For review see; Ogbunude and Baer, 1993). Two distinct NT processes that (i) exhibit either purine- or pyrimidine-nucleoside selectivity with an overlapping selectivity for adenosine, and (ii) are insensitive to inhibition by NBMPR and dipyrindamole, are present in the Trypanosomatidae *Leishmania donovani* (K_m values: 1-5 μ M; Aronow *et al.*, 1987; Ogbunude *et al.*, 1991) and *Trypanosoma cruzi* (Finley *et al.*, 1988). *Trypanosoma brucei* also expresses a dipyrindamole-sensitive transport process (K_m value: <1 μ M) that accepts both adenine and adenosine as permeants (Carter and Fairlamb, 1993). The protozoan *Trichomonas vaginalis* expresses two NT processes (K_m values: 4-14 μ M)

similar to those described above except that both processes accept adenosine and uridine as permeants (Harris *et al.*, 1988).

Trophozoites of *Giardia intestinalis* possess two NT processes (K_m values; low micromolar range): one that accepts all nucleosides, except thymidine as permeants, and a second that is specific for thymidine (Davey *et al.*, 1992; Ey *et al.*, 1992). Interestingly, Davey *et al.* (1992) showed that the *G. intestinalis* broad-specificity NT process shares with the mammalian *es* NT process a requirement for the 3'-hydroxy substituent of the nucleoside.

Intraerythrocytic parasites have been demonstrated to induce or confer NT processes in erythrocyte plasma membrane unlike the *es* NT process normally present. Studies of *Plasmodium yoelii*- and *Plasmodium falciparum*-infected erythrocytes have demonstrated the presence of two parasite-related NT processes, in addition to the native *es* NT process of the erythrocyte (Gati *et al.*, 1987; Gati *et al.*, 1990; Gero *et al.*, 1987; Gero *et al.*, 1989; Gero and Upston, 1992). The parasite-related NT processes apparently consist of a saturable NBMPR-insensitive NT process and a second non-saturable transport process. Gati *et al.* (1990) have demonstrated that these parasite-related transport processes accept both D- and L-enantiomers of adenosine as permeants, unlike the *es* NT process which exhibits selectivity for D-adenosine (Gati *et al.*, 1989). The non-saturable transport process may be similar or identical to the recently described nutrient-permeable channel expressed in *P. falciparum*-infected erythrocytes (Desai *et al.*, 1993). Bovine erythrocytes, which normally lack a NT process, have been shown to express a NT process that is sensitive to inhibition by micromolar concentrations of NBMPR, dilazep, and dipyridamole upon infection with the parasite *Babesia bovis* (Gero, 1989). The inhibitor sensitivity profile of the *B. bovis*-induced NT process resembles that of the mammalian *ei* NT process, however their relatedness has yet to be determined.

Nucleoside uptake has been reported in the helminth *Schistosoma mansoni* (Levy and Read, 1975; El Kouni and Cha, 1987; El Khouni *et al.*, 1987; El Kouni *et al.*, 1989) and a saturable, Na^+ -dependent nucleoside uptake process(es) (K_m values: 100-210 μM) has been shown to exist in *Hymenolepis diminuta* (Page and MacInnis, 1975; McCracken *et al.*, 1975). Interpretation of these results is complicated by the use of intact worms in uptake studies, which likely reflect a combination of nucleoside transport (across one or more membranes) and metabolism processes.

MATERIALS AND METHODS

Materials

D-[5,6- ^3H]Uridine, D-[2,8- ^3H]adenosine, D-[methyl- ^3H]thymidine, [^3H]H $_2\text{O}$, and [U- ^{14}C]sucrose were obtained from ICN Biomedicals; [G- ^3H]NBMPR was from Moravsek Biochemicals; L-[5- ^3H]Uridine, L-[2,8- ^3H]adenosine, L-[methyl- ^3H]thymidine, and non-radioactive L-nucleosides were generously provided by A. R. P. Paterson (University of Alberta); paraffin oil (Saybolt Viscosity 125-135) was from Fisher Scientific; silicon 550 oil was from Dow Corning Corp.; dilazep dihydrochloride was a gift from Hoffman La Roche & Co. (Basel, Switzerland); fetal bovine serum and plastic culture flasks were from Gibco laboratories, and Excell 400 media was from J.R.H. Biosciences (Woodland, CA); All other chemicals were obtained from standard commercial sources.

Cell Culture

Spodoptera frugiperda ovary (Sf9) cells were obtained from ATCC (#1711-CRL) and were maintained as static cultures in sealed plastic flasks at 27°C in Excell 400 media that contained 1% fetal bovine serum. Concentrations of cells in cultures were determined using a Coulter Counter Model ZF electronic particle counter (Coulter Electronics Inc.). Stock cultures were grown to densities of 1×10^6 to 2×10^6 cells/ml and were subcultured every 2-3 days to a density of 1×10^5 - 2×10^5 cells/ml. Cell cultures were maintained for a maximum of 20-30 generations. Large scale cultures were grown in the same media in appropriately-sized glass spinner flasks (Bellco) with constant mixing (≈ 60 rpm).

Measurement of Nucleoside Influx

a) Buffers and Solutions Used in Transport Assays

i. Transport Oil

The oil mixture utilized in transport assays was prepared by mixing approximately 75 ml paraffin oil (Saybolt Viscosity 125-135) with 425 ml silicon 550 oil. The final specific gravity (Specific Gravity/Baume Hydrometer) was adjusted to 1.032 g/ml using either type of oil.

ii. Nucleoside, Nucleobase, and NT Inhibitor Solutions

Sterile stock solutions of non-radioactive nucleobases, nucleosides, and NT inhibitors were prepared by dissolving the compound in sterile distilled H₂O and filtering through a sterile 0.2 μ m Millex-GV filter (Millipore). All solutions were stored at -20°C. Concentration of stock solutions was determined by UV absorbance using the molar extinction coefficient ($\bar{\epsilon}$) of the compounds at a wavelength of maximum absorption ($\lambda_{\max}$), as listed below. The concentration of working solutions, created by dilution of stock solution, was ascertained by UV absorbance prior to use. Radiolabeled nucleosides were purified with a Varian 5000 high-pressure liquid chromatograph using water-methanol gradients on a C18 reverse phase column, as described in detail elsewhere (Boleti, 1991). Purified nucleosides were stored as water-methanol solutions at 4°C and were used within 2 months of the purification date.

Compound	$\lambda_{\max}$ (nm)	$\bar{\epsilon}$ (mM ⁻¹ cm ⁻¹)
Ade	260.5	13.4
Ado	259	15.4
dAdo	259	15.4
Dilazep	266	18.42
dThd	267	9.73
dUrd	262	10.1
Formycin B	216	13.14
Guo	252	13.7
Ino	249	12.2
NBMPR	290	25.1
Ura	260	8.2
Urd	262	10.1

iii. Bug Transport Buffer (BTB)

These media, based upon Graces Insect medium (Grace, 1962), were adjusted to pH 6.2 and had an osmolality of ≈ 300 mOsm (Wescor Vapor Pressure Osmometer,

Model 5500). The Na⁺-free BTB was generated by the substitution of NaHCO₃ and NaH₂PO₄ with equimolar amounts of KHCO₃ and KH₂PO₄, respectively.

BTB	Sodium-Free BTB
7.0 mM CaCl ₂	7.0 mM CaCl ₂
55.0 mM KCl	55.0 mM KCl
11.0 mM MgSO ₄	11.0 mM MgSO ₄
11.0 mM MgCl ₂	11.0 mM MgCl ₂
4.0 mM glucose	4.0 mM glucose
78.0 mM sucrose	78.0 mM sucrose
4.0 mM NaHCO ₃	4.0 mM KHCO ₃
7.0 mM NaH ₂ PO ₄	7.0 mM KH ₂ PO ₄

b) Measurement of Zero-Trans Nucleoside Influx

Cells were harvested by centrifugation (100 x g, 5 min) and resuspended in transport buffer at 0.5×10^7 - 1.0×10^7 cells/ml. Suspensions of cells were utilized within 30 min of harvesting from growth medium and influx assays were conducted at RT ($\approx 22^\circ\text{C}$). Where necessary, cell density was determined by counting cells from replicate 100 μl aliquots using a Coulter Counter. Portions (100 μl) of transport medium containing ³H-nucleoside were layered over 200 μl portions of transport oil in 1.5 ml microcentrifuge tubes. The tubes were placed in the rotor of a microcentrifuge (Eppendorf Models 5412 or 5414) and influx intervals were initiated by addition of 100 μl portions of cell suspension. Influx intervals were stopped by centrifugal pelleting (9000 x g, ≥ 30 sec) of cells under the oil layer; hereafter referred to as the "oil-stop" method. By adding portions of cell suspensions successively to microcentrifuge tubes placed in a single rotor, it was possible to conduct a maximum of 6 uptake assays simultaneously. Metronome signals were used to time the addition of cells to each of these six tubes at intervals separated by 2 sec. The majority of influx assays conducted in this study employed a "inhibitor-stop" method as an alternative to the "oil-stop" procedure. In the "inhibitor-stop" method, the period of influx was terminated by the addition of 200 μl of ice-cold transport buffer that contained 200 μM dilazep, followed immediately by centrifugation (9000 x g, ≥ 30 sec) of cells under the oil layer. Measurement of influx at each interval was conducted in triplicate. Aqueous layers in the assay tubes were removed and the tube walls were washed twice with water. The oil layers were then removed and the cell pellets were dissolved in 250 μl of 5% Triton X-100 by vortexing. The tubes were placed in scintillation vials, 5 ml Ecolite scintillant was added and radioactive content was measured.

Total cell pellet and extracellular pellet volumes were determined, respectively, by substituting [³H]H₂O and [¹⁴C]sucrose for ³H-nucleoside in the influx assay. Intracellular pellet volume was calculated as the difference between total and extracellular volume determinations. Radioactivity associated with cell pellets was determined by liquid scintillation counting and all assays were conducted in triplicate.

c) Calculation of Initial Rates and Kinetic Parameters

Zero-trans influx, which represents the rate of transfer of nucleoside molecules from the *cis* to the *trans* face of the plasma membrane, were calculated from (i) linear time courses of uptake by linear regression using the least squares method, and (ii) non-linear time courses by second-order polynomial regression using the least squares method (Hewlett-Packard HP-41C calculator with Stat Pac). For a single NT process, zero-trans fluxes reach a limiting maximum velocity as concentration increases. The relationship between influx (V) and permeant concentration (S) can be described by $V = (S \cdot V_{\text{max}})/(K_m + S)$ (Equation 1), wherein V_{max} and K_m represent, respectively, the

maximal limiting influx and permeant concentration at the *cis* face that produces the half maximal influx (Stein, 1986). Equation 1 is mathematically identical to the Michaelis-Menten equation and kinetic parameters of influx can be estimated from linear transformations of the data according to the method of Rosenthal (1967). In cases of simultaneous expression of two kinetically distinct processes, Equation 1 can be modified to $V_t = V_1 + V_2 = [(S \cdot V_{\max 1}) / (K_m 1 + S) + (S \cdot V_{\max 2}) / (K_m 2 + S)]$ (Equation 2), wherein V_t is total flux, V_1 and V_2 are fluxes for process 1 and 2, $V_{\max 1}$ and $V_{\max 2}$ are maximal fluxes for processes 1 and 2, and $K_m 1$ and $K_m 2$ are the permeant concentrations at the *cis* face at half $V_{\max 1}$ and $V_{\max 2}$, respectively. Rosenthal transformation of such data, graphically represented as V_t/S versus V_t , results in a non-linear plot. In this study, influx of a given nucleoside was simultaneously mediated by two distinct processes, hence the K_m and $V_{\max}$ values were estimated by limited slope analysis (Hunston, 1975). This technique utilizes experimentally derived slopes and axis intercepts to estimate limiting slopes and intercepts on the Rosenthal plot. Kinetic parameters (K_m and $V_{\max}$ values) of nucleoside transport were also fit to a two transporter model using iterative curve fitting by non-linear regression analysis using the UltraFit curve-fitting program (Biosoft, Cambridge, U.K.).

d) Inhibition of Nucleoside Influx

The effects of NT inhibitors on nucleoside influx were assessed using a procedure that minimized depletion of free inhibitor. Cells were incubated at $\approx 1 \times 10^6$ cells/ml for 5 min in BTB that contained graded concentrations of inhibitor. Portions of cell suspensions (100 μ l) were then used in influx assays as described above. The ^3H -nucleoside solutions used in the assay also contained inhibitor at the same concentrations as were present in the cell suspensions.

Equilibrium Binding of [^3H]NBMPR to Intact Sf9 Cells

Binding of NBMPR to Sf9 cells was determined using a modification of an established method (Cass *et al.*, 1981). Cells were harvested by centrifugation (100 x g, 5 min) and washed once and resuspended in BTB at 2×10^6 cells/ml. Portions (500 μ l) of cell suspension were added to 500 μ l of BTB containing graded concentrations of [^3H]NBMPR alone or with 20 μM nitrobenzylthioguanosine (NBTGR) that had been layered over 250 μ l of transport oil in microcentrifuge tubes. The mixtures were incubated 20 min at RT ($\approx 22^\circ\text{C}$). Cells were centrifugally pelleted and samples were processed thereafter as described for influx assays. The concentration of free NBMPR was estimated from the radioactive content of the aqueous medium after centrifugation and the quantity of total and non-specifically bound NBMPR was determined from the radioactive content of cell pellets incubated in the absence and presence of NBTGR, respectively.

RESULTS

Nucleoside Transport Processes Exhibit Enantiomeric Selectivity

Studies with mammalian cells have established that at least three NT processes (*es*, *ei*, *cif*) exhibit stereoselectivity for the D-enantiomer of various nucleosides (Gati *et al.*, 1989; Paterson *et al.*, 1991). The extent to which stereoselective, and thus mediated, processes contributed to total nucleoside influx in Sf9 cells was assessed by determining initial rates of uptake of 25 μM ^3H -labeled L- and D-enantiomers of adenosine, thymidine and uridine under zero-*trans* conditions. Results shown in Fig. V 1 reveal that

accumulation of the D-enantiomers occurred at constant rates from 0 to 20 sec, whereas influx of the corresponding L-enantiomers was negligible over this period. The small amount of L-nucleoside accumulated by Sf9 cells during the 20 sec incubations indicated that the L-enantiomers were poor permeants at best and that diffusional permeation contributed only slightly to total fluxes.

Sf9 Cells Express a Component of Non-Specific Nucleoside Association

Assuming the L-nucleosides not to be permeants, the association of nucleoside with cells in the absence of mediated influx, as shown in Fig. V 1, was approximated by extrapolation of time courses obtained for mediated (D-enantiomer) and non-mediated (L-enantiomer) influx of a nucleoside to their points of intersection on the x axis. A non-transport related association of nucleoside with Sf9 cells was suggested by the observation that substantial amounts of L- and D-nucleoside (2-2.5 pmol/ μ l cell H₂O and 5 - 8 pmol/ μ l cell H₂O, respectively) were associated with cells at the "experimental time zero" as well as at their points of intersection (2-2.5 pmol/ μ l cell H₂O). To assess the nature of this apparently non-transport related phenomenon, the amount of nucleoside associated with cells in the absence of mediated nucleoside influx was further examined. The experiment of Fig. V 2A examined the effectiveness of an "inhibitor-stop" method used previously in which ice-cold buffer containing 200 μ M dilazep was added to reaction mixtures to instantaneously block fluxes mediated by the mammalian *es*, *ei*, and *cif* NT transporters (Hogue *et. al.*, 1990; Boleti, 1991). Time courses for Sf9 influx of 25 μ M [³H]thymidine were produced using both the oil-stop and inhibitor-stop methods, and the total amounts of cell-associated radioactivity were plotted as a function of the time of initiation of centrifugation (oil-stop) or addition of quenching solution (inhibitor-stop). The rate of influx obtained with the two methods were the same (1.6 pmol/[μ l cell H₂O-s]). The total amounts of cell-associated radioactivity obtained with the two methods differed by an amount equivalent to that accumulated during 2-3 sec, the period of time shown previously (Harley *et. al.*, 1982) to be sufficient to completely remove cells from the ³H-permeant solution by the oil-stop method. Instantaneous and sustained inhibition of flux by the inhibitor-stop method was demonstrated by adding the ice-cold dilazep-containing solution after 4 sec of exposure to [³H]thymidine and centrifuging after periods of incubation. Similar results (not shown) were obtained when [³H]uridine and [³H]adenosine were used as permeants. Since mediated nucleoside influx was completely inhibited using the inhibitor-stop method, the amount of nucleoside associated with cells at the inhibitor-stop "time zero" was assumed to reflect a non-transport related association of nucleoside.

The nature of this non-transport related association of nucleoside was addressed further by an examination of the concentration dependence of the association of [³H]adenosine or [³H]uridine with Sf9 cells in the presence of ice-cold solutions that contained both permeant and 200 μ M dilazep. Fig. V 2B shows that the quantity of nucleoside associated with cells increased linearly with concentration of nucleoside. This phenomenon was not due to a general increase in permeability since the extracellular pellet volumes remained constant (22 $\pm$ 3 % of total pellet volume) during this experiment. This apparent non-saturable association of nucleosides with Sf9 cells accounted for approximately 20% of total radioactivity accumulated by cells during the 10 sec influx periods used for much of this study. This association was constant over time for a given concentration of nucleoside and thus did not interfere with the analysis of initial rates of influx.

High- and Low-Affinity Transport Processes Mediate the Influx of Adenosine and Uridine

The kinetic parameters of the NT processes of Sf9 cells were estimated by determining the concentration dependence of influx of [^3H]uridine and [^3H]adenosine (1–1000 μM), respectively, under zero-*trans* conditions. Velocities of influx determined in two separate experiments gave rise to non-linear Rosenthal plots (Rosenthal, 1967), as shown in Fig. V 3. These results suggested the presence of high- and low-affinity transport processes for both permeants, hence the data were fit to a two-transporter kinetic model. The values (mean $\pm$ S.D. for two experiments) for adenosine transport were: high-affinity process, 10 ± 4 μM (K_m) and 5 ± 2 pmol/ $[\mu\text{l cell H}_2\text{O}\cdot\text{s}]$ (V_{max}); low-affinity process, 440 ± 140 μM (K_m) and 39 ± 6 pmol/ $[\mu\text{l cell H}_2\text{O}\cdot\text{s}]$ (V_{max}). The values for uridine transport were: high-affinity process, 43 ± 6 μM (K_m) and 5 ± 1 pmol/ $[\mu\text{l cell H}_2\text{O}\cdot\text{s}]$ (V_{max}); low-affinity process, 1121 ± 111 μM (K_m) and 83 ± 21 pmol/ $[\mu\text{l cell H}_2\text{O}\cdot\text{s}]$ (V_{max}).

High-Affinity Transport is Mediated by Distinct Purine- and Pyrimidine-Nucleoside Selective Processes and a Single Low-Affinity Transport Accepts Both Purine- and Pyrimidine-Nucleosides

Adenosine and uridine appear to be universal permeants of the nucleoside transporters of mammalian cells and have been used extensively in competition studies to assess permeant selectivity. Influx of 1 μM [^3H]adenosine and [^3H]uridine, a concentration at which influx would be mediated only by the high-affinity process(es), was examined in the presence of increasing concentrations (0 to 1000 μM) of various purine and pyrimidine nucleosides, nucleobases, and ribose. As shown in Fig. V 4, influx was specific for nucleosides since the two bases tested (uracil, adenine) inhibited influx only at very high concentrations, and ribose had no effect at any of the concentrations tested. Influx of [^3H]uridine was inhibited, in a concentration-dependent manner, by the pyrimidine nucleosides tested (uridine > 2'-deoxyuridine > thymidine) but not by adenosine, whereas influx of [^3H]adenosine was inhibited by the purine nucleosides (adenosine > 2'-deoxyadenosine > formycin B > guanosine > inosine) and only slightly by uridine. Thus, this pattern of inhibition suggested the presence in Sf9 cells of two functionally distinct high-affinity NT processes, with selectivity for either pyrimidine nucleosides or purine nucleosides. The influx observed in the presence of 1 mM non-radioactive uridine and adenosine was likely due to diffusional entry and non-specific association of nucleosides.

Permeant selectivity of the low-affinity NT process(es) was assessed by measuring influx of 100 μM [^3H]adenosine and [^3H]uridine, a concentration in excess of that required to saturate the high-affinity NT processes, in the presence of increasing concentrations (0–2 mM) of adenosine and uridine. These results are shown in Fig. V 5. Influx of [^3H]uridine was inhibited equally well, in a concentration-dependent manner, by both uridine and adenosine, whereas [^3H]adenosine influx was more efficiently inhibited by lower concentrations of adenosine rather than uridine. The decreased concentration-dependent inhibition of [^3H]adenosine influx by uridine is consistent with the apparent higher K_m value for uridine (≈ 1.1 mM) versus adenosine (≈ 0.4 mM) exhibited by the low-affinity NT process. These observations suggest that Sf9 cells also possess a single transport process with relatively low affinity for both purine and pyrimidine nucleosides.

The High-Affinity NT Processes are Insensitive to Inhibition by NBMPR, Dilazep, and Dipyridamole

NBMPR, dilazep and dipyridamole are potent inhibitors of the equilibrative NT processes of mammalian cells that have proven useful in distinguishing between functionally distinct transporter subtypes. At nM concentrations, NBMPR inhibits only *es* transporters whereas dipyridamole and dilazep inhibit both *ei* and *es* transporters. The sensitivity of *zero-trans* influx of 1 μ M uridine and adenosine to these three agents was examined to ascertain the relatedness of the high-affinity NT processes to the mammalian equilibrative NT processes. The results shown in Fig. V 6 reveal that none of the inhibitors produced significant reductions in uridine or adenosine influx until inhibitor concentrations of $>1 \mu$ M were reached. The general insensitivity of the high-affinity NT processes of Sf9 cells towards these inhibitors suggest that structural differences exist between these and the mammalian equilibrative NT processes.

The slight inhibition of influx (5-10%) seen in the experiment of Fig. V 6 when Sf9 cells were exposed to low concentrations (1-10 nM) of NBMPR suggested the presence of a small amount of *es*-like NT activity. To examine this possibility, the binding of [^{3} H]NBMPR to intact Sf9 cells under equilibrium conditions was measured. Fig. V 7 illustrates that the amount of total and non-specifically bound [^{3} H]NBMPR was similar over a concentration range normally used to measure high-affinity NBMPR binding sites in mammalian cells. These results confirmed the absence of any *es*-like NT activity. It was of interest that the apparent non-saturable nature of [^{3} H]NBMPR binding resembled that of the non-transport related association of nucleoside observed in Fig. V 2B.

The High-Affinity NT Processes Function Via a Na⁺-Independent and Equilibrative Mechanism

The Na⁺-dependent, concentrative NT processes of mammalian cells are not inhibited by NBMPR, dilazep or dipyridamole. The high-affinity NT processes of Sf9 cells exhibited a similar insensitivity to these inhibitors, therefore long-term uptake assays were conducted in Na⁺-free buffer to ascertain the dependence of NT on a plasma membrane Na⁺ gradient. Fig. V 8 shows that substitution of K⁺ for Na⁺ did not significantly alter the uptake of either 10 μ M uridine or adenosine over 5 min, a condition under which influx would be largely mediated by the high-affinity NT processes. It appears the high-affinity NT processes observed in Sf9 cells were largely Na⁺-independent and thus probably functioned by an equilibrative mechanism.

Formycin B is a non-metabolized analog of adenosine that has been used to ascertain the equilibrative or concentrative nature of NT processes of mammalian cells (Dagnino, 1988; Plagemann and Woffendin, 1989; Vijayalakshmi *et. al.*, 1992). In the competition experiments of Fig. V 4, formycin B inhibited influx of adenosine but not of uridine, a result that suggested that formycin B was a permeant of the purine-selective process. Shown in Fig. V 9 are results from measurements of cell-associated formycin B during a 5 min exposure of Sf9 cells to 6 μ M and 400 μ M [^{3} H]formycin B. The apparent cellular concentration of formycin B (pmol/ μ l cell H₂O = μ M) rapidly approached, but did not exceed, the extracellular concentration. These results are consistent with high- and low-affinity transport of purine nucleosides being mediated by equilibrative, rather than concentrative, mechanisms. A non-metabolized ^{3} H-labeled pyrimidine nucleoside was not available to conduct parallel studies of the pyrimidine-selective NT process of Sf9 cells.

DISCUSSION

This study of nucleoside permeation in Sf9 cells has revealed the presence of NT processes that share some features with their mammalian counterparts, but that also represent a unique class of transporters not previously described. Table V 2 illustrates the similarities and differences between the functional characteristics of known mammalian NT processes and the high - affinity NT processes of Sf9 cells. The low-affinity NT process of Sf9 cells was not characterized to the extent of the high-affinity processes because experimental conditions under which only the low-affinity process was functional could not be easily generated.

The enantiomeric configuration of the ribofuranosyl moiety is an important determinant for acceptance as a permeant by mammalian nucleoside transporters. Mouse cells expressing *es* NT (erythrocytes) or simultaneously expressing *es*, *ei*, and *cif* NT (cultured L1210/AM cells) possess transporter stereoselectivity for the D-enantiomer of adenosine (Gati *et al.*, 1989); in this study, the small amount of L-adenosine influx was attributed to both diffusion and acceptance as a poor substrate for one or more of the NT processes of L1210 cells. Sf9 cells also displayed selectivity for the D-enantiomers, suggesting that stereoselectivity may be a conserved characteristic among nucleoside transporters of eucaryotic cells. The low fluxes of L-nucleoside influx observed in this study may have been due to mediated permeation as well as diffusion, since the L-nucleosides appeared to be poor permeants for the nucleoside transporters of Sf9 cells.

The presence of a substantial cellular association of nucleoside permeant at time zero, as was observed for adenosine and uridine association with Sf9 cells, has not been described in similar studies with mammalian cells. This non-saturable association is distinct from mediated nucleoside transport processes present in Sf9 cells and may reflect low-affinity binding or adsorption of nucleosides to the plasma membrane, however this has yet to be determined.

Analysis of the kinetic parameters of zero-*trans* influx of either uridine or adenosine revealed the presence of both high- and low-affinity NT processes in Sf9 cells that exhibit an approximate 20-fold difference in K_m values, depending on the permeant in question. Similarly, a number of mammalian cell types simultaneously express equilibrative NT processes of different permeant affinities. For example, the *es* and *ei* NT processes of human K562 cells exhibit high and low permeant affinities, respectively, with K_m values that differ 3- to 4-fold (Boleti, 1991). A multiplicity of NT processes has also been observed in many non - mammalian eucaryotic cell types.

Nucleoside competition studies that utilized 1 μ M [^{3} H]adenosine or [^{3} H]uridine, a concentration at which influx would be mediated by only the high-affinity NT processes, suggested two distinct routes of nucleoside influx in Sf9 cells with little, if any, overlap in permeant selectivity. The two high-affinity processes exhibited absolute selectivity for purine or pyrimidine nucleosides, a characteristic that has not been seen in studies with any of the known eucaryotic transporters. Although a preference for purine or pyrimidine nucleosides was observed for two distinct NT processes of *Trypanosoma cruzi* and *Leishmania donovani*, adenosine was accepted as a permeant by both processes (Finley *et al.*, 1988; Aronow *et al.*, 1987). The low-affinity NT process of Sf9 cells mediated influx of both uridine and adenosine, a characteristic exhibited by all of the mammalian NT processes identified thus far.

The demonstration that formycin B transport via the purine-selective NT process was likely equilibrative is consistent with the observation that transport was independent of a plasma membrane Na^+ gradient. It is likely that the pyrimidine-selective NT process, which was Na^+ -independent, functioned in a similar manner. The observation that a high concentration of formycin B was transported in an equilibrative manner suggested the low-affinity NT process functioned in an equilibrative manner, however formycin B was not directly demonstrated to be a permeant of this process. The exact

functional nature of these transport processes will be ascertained only by characterization of each NT process when expressed individually.

Since influx of 1 μ M nucleosides in Sf9 cells was unaffected by low (≤ 100 nM) concentrations of NBMPR, the two high-affinity processes were resistant to inhibition by NBMPR. The low sensitivity of the NT processes of Sf9 cells to these transport inhibitors is not unique since *Leishmania donovani*, *Trypanosoma cruzi*, and *Aedes albopictus* (mosquito) cells also exhibit NT processes that are insensitive to low concentrations of NBMPR, dipyrindamole, and/or dilazep (Aronow *et al.*, 1987; Finley *et al.*, 1988; Abidi *et al.*, 1987). Thus, it appears that the relationship between the action of tight-binding NT inhibitors and the equilibrative mechanisms exhibited by mammalian *es* and *ei* NT processes is not maintained in many cases of non-mammalian eucaryotic NT.

In conclusion, our studies have revealed the presence of unique purine- and pyrimidine-selective equilibrative NT processes in cultured *S. frugiperda* (Sf9) cells. These high-affinity NT processes are kinetically similar to those exhibited by mammalian cells however, their combination of permeant selectivity and inhibitor sensitivity constitutes a new class of NT processes distinct from that observed in any other eucaryotic cell studied to date. The low-affinity NT process of Sf9 cells generally resembles the inhibitor-insensitive NT processes exhibited by both mammalian and non-mammalian cell types. The multiplicity of NT processes present in Sf9 cells will clearly complicate any future studies involving the functional characterization of a recombinant mammalian NT protein in this cell type.

TABLE V 1

Kinetic Parameters for Nucleoside Transport Processes in Sf9 Cells

Velocities of nucleoside transport at RT (22°C) were derived from time courses of ³H-nucleoside uptake in Sf9 cells using the inhibitor-stop method. Adenosine and uridine were employed as pyrimidine and purine nucleoside permeants, respectively. Transport velocities of at least 8 concentrations of each nucleoside, ranging from 1-1000 µM, was examined in two separate experiments conducted on different days. Kinetic parameters were derived by (i) graphical analysis of Rosenthal plots (velocity versus velocity/concentration) using the limited slope analysis (LSA) method of Hunston (1975) or (ii) by non-linear regression analysis (NLRA) using the UltraFit curve-fitting program. The mean values ($\pm$ SD) for the two experiments as determined by both methods, are presented in bold type.

	Adenosine Transport				Uridine Transport			
	<u>High - Affinity</u>		<u>Low - Affinity</u>		<u>High - Affinity</u>		<u>Low - Affinity</u>	
	Km (μ M)	Vmax (μ M/sec)	Km (μ M)	Vmax (μ M/sec)	Km (μ M)	Vmax (μ M/sec)	Km (μ M)	Vmax (μ M/sec)
<u>Experiment 1</u>								
LSA	4.2	2.5	464	45	38.6	4.6	500	59
NLRA	9.5	4.4	370	40	ND	ND	ND	ND
<u>Experiment 2*</u>								
LSA	12.4	5.6	294	31	49.5	4.3	1042	97
NLRA	13.8	6.6	621	39	41.3	6.1	1199	95
	10.0 ± 4.2	5.5 ± 1.1	437 ± 141	39 ± 6	43.1 ± 5.6	5.0 ± 1.0	914 ± 366	84 ± 21

*Values are from experiment presented in Figure V 3. ND; not determined (goodness of fit index was ≤ 0.99 and computer-generated values were deemed unacceptable).

TABLE V 2

Properties of Nucleoside Transporter Subclasses

Classification adapted from Vijayalakshmi and Belt (1988) and Belt (1992). nd = not determined. Abbreviations: *pur*, purine-selective; *pyr*, pyrimidine-selective.

Classification:	Mammalian					Sf9	
	equilibrative		concentrative			equilibrative	
	<i>es</i>	<i>ei</i>	<i>cif</i>	<i>cit</i>	<i>cib</i>	<i>pur</i>	<i>pyr</i>
D-enantiomer selective	+	+	+	nd	nd	+	+
Na ⁺ -dependent	-	-	+	+	+	-	-
Permeants:							
adenosine	+	+	+	+	+	+	-
guanosine	+	nd	+	-	+	+	-
formycin B	+	+	+	-	+	+	-
uridine	+	+	+	+	+	-	+
thymidine	+	+	-	+	+	nd	+
Inhibited by :							
NBMPR	+	-	-	-	-	-	-
dipyridamole	+	+	-	-	-	-	-
dilazep	+	+	-	-	-	-	-

FIGURE V 1

Nucleoside Influx in Sf9 cells is Mediated by Stereoselective Processes

Actively proliferating Sf9 cells were suspended in BTB (1.7×10^7 cells/ml) and used in influx assays that employed the oil-stop method, as detailed in Materials and Methods. Portions of cell suspension (100 μ l) were added to 100 μ l portions of 50 μ M [^{3} H]uridine (■,□), [^{3} H]adenosine (●,○), or [^{3} H]thymidine (▲,△) that had been layered over 200 μ l of transport oil in microcentrifuge tubes. Influx intervals were terminated by initiating centrifugation after varying periods of incubation at RT ($\approx 22^\circ\text{C}$). Data for the D- (■,●,▲) and L- (□,○,△) stereoisomers of each nucleoside are expressed as mean values $\pm$ SD; error bars are not shown where SD values were smaller than the size of data symbols.

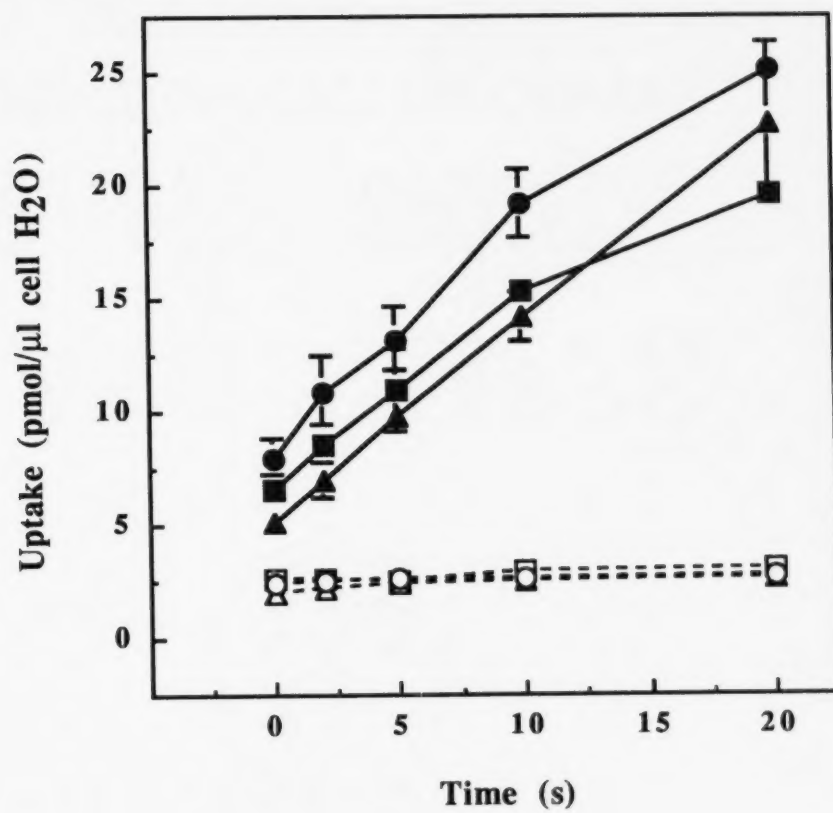


FIGURE V 2

The Inhibitor-Stop Method for Determining Uptake of Nucleosides Can Define a Component of Non-Specific Nucleoside Association in Sf9 Cells

Panel A: Thymidine flux is instantaneously inhibited using the inhibitor-stop method. Cellular uptake of 25 μM [^3H]thymidine was determined by the oil-stop method described in Figure V 1 (●) or by the inhibitor-stop method described in Materials and Methods. The inhibitor-stop method involved the addition of 200 μl of ice-cold BTB containing 0.2 mM dilazep to assay mixtures at the indicated time points, followed immediately thereafter by centrifugation through oil in the same manner as described for the oil-stop procedure (■). For some samples, 200 μl ice-cold BTB containing 0.2 mM dilazep was added to assay mixtures after 4 sec of influx and the cells were centrifuged through oil 1, 2, 3, and 5 sec later (□). Data are expressed as mean $\pm$ SD, and error bars are not shown where SD values were smaller than the size of data symbols. Velocity of thymidine influx was calculated as 1.6 pmol/[μl cell $\text{H}_2\text{O} \cdot \text{s}$] using either oil-stop or inhibitor-stop method.

Panel B: Nucleoside is associated with cells in the absence of mediated influx. The association of graded concentrations of [^3H]adenosine (○) or [^3H]uridine (□) with cells at zero time was determined using the inhibitor-stop method. Cells in BTB (100 μl , 2×10^7 cells/ml) were layered over oil (200 μl), ice-cold BTB containing 0.2 mM dilazep (200 μl) and 1 sec later BTB containing ^3H -nucleoside (100 μl) was added. The mixture was then immediately centrifuged. Data are expressed as mean $\pm$ SD, and error bars are not shown where SD values were smaller than the size of data symbols. The extracellular volume of total cell pellet volume measured in this experiment was calculated as $22 \pm 2\%$.

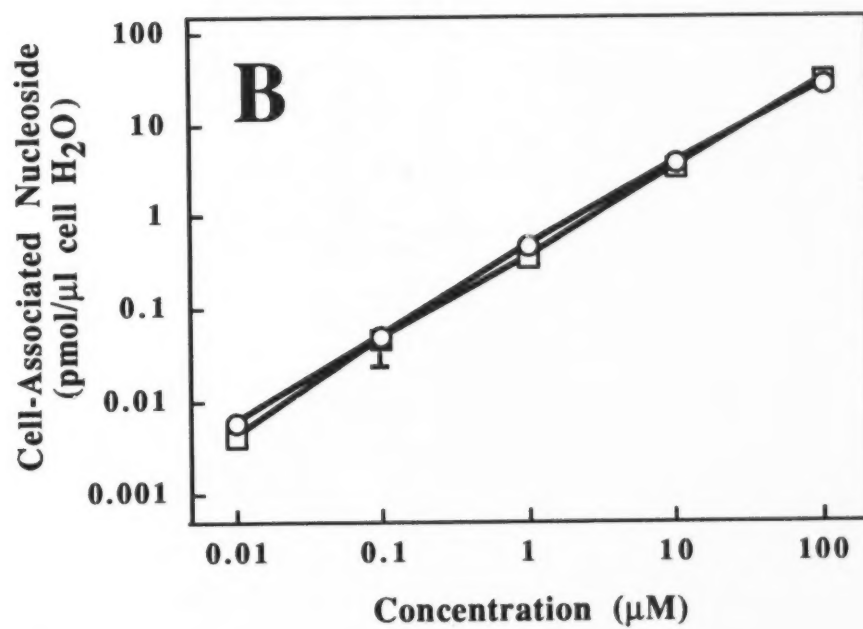
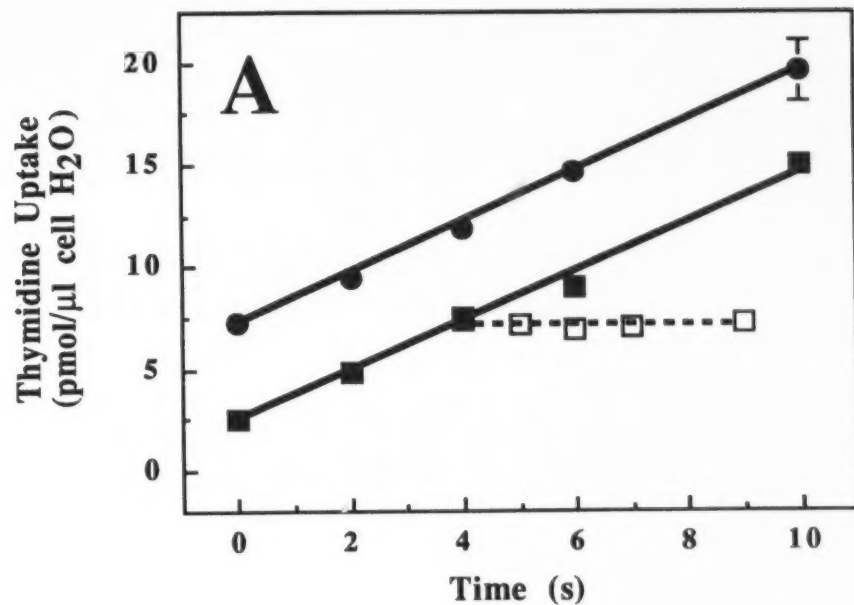


FIGURE V 3

Nucleoside Influx in Sf9 Cells is Mediated by High- and Low-Affinity Processes

Initial transport velocities at RT ($\approx 22^{\circ}\text{C}$) were determined using actively proliferating Sf9 cells. Time courses (0-6 sec) of zero-trans [^3H]adenosine and [^3H]uridine were obtained over a 1-500 μM concentration range using the inhibitor-stop method described in Materials and Methods. Transport velocities were calculated by either linear regression or polynomial regression using the least squares method. Data from a single representative experiment, presented as a Rosenthal plot of initial rates of transport as a function of transport rate/nucleoside concentration, are shown. The kinetic parameters from two separate experiments were determined according to a two transporter model using iterative curve fitting by regression analysis and also by limited slope analysis (Hunston, 1975). The values (mean $\pm$ SD for two experiments) for adenosine transport were: high-affinity process, $10 \pm 4 \mu\text{M}$ (K_m) and $5 \pm 2 \text{ pmol}/[\mu\text{l cell H}_2\text{O}\cdot\text{s}]$ (V_{max}); low-affinity process, $440 \pm 140 \mu\text{M}$ (K_m) and $39 \pm 6 \text{ pmol}/[\mu\text{l cell H}_2\text{O}\cdot\text{s}]$ (V_{max}). The values for uridine transport were: high-affinity process, $43 \pm 6 \mu\text{M}$ (K_m) and $5 \pm 1 \text{ pmol}/[\mu\text{l cell H}_2\text{O}\cdot\text{s}]$ (V_{max}); low-affinity process, $1121 \pm 111 \mu\text{M}$ (K_m) and $83 \pm 21 \text{ pmol}/[\mu\text{l cell H}_2\text{O}\cdot\text{s}]$ (V_{max}).

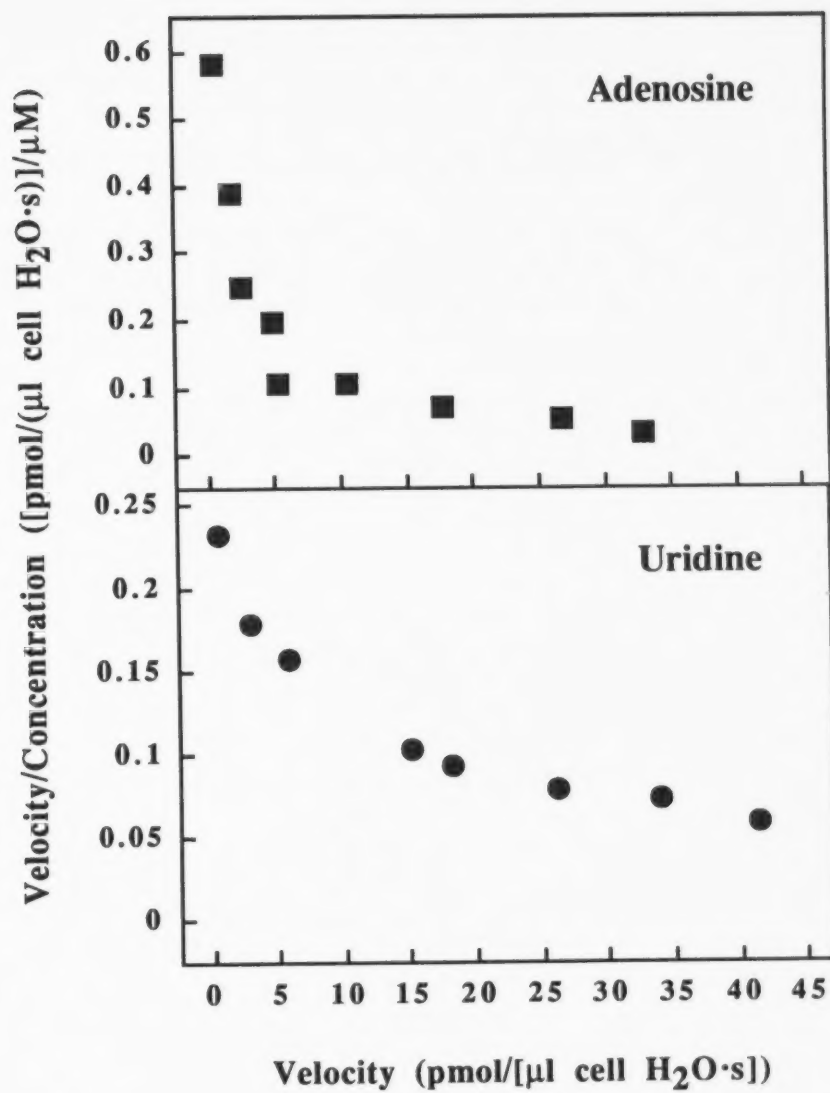


FIGURE V 4

High-Affinity Transport of Nucleosides is Mediated by Distinct Purine- and Pyrimidine-Nucleoside Selective Processes

Portions of Sf9 cells (100 μ l, 1.5×10^7 cells/ml) were incubated at RT ($\approx 22^\circ\text{C}$) with 100 μ l of BTB that contained 2 μ M [^3H]uridine (A) or [^3H]adenosine (B) together with increasing concentrations of potential competitive substrates. Influx assays were terminated after 10 sec of incubation, a period over which linear time courses of influx were observed, using the oil-stop method. Influx in the presence of competing substrates are expressed as percent of flux in their absence (controls). Values are the means of three determinations (SD values were $\leq 5\%$ and thus are not shown). Abbreviations: Ade, adenine; Ado, adenosine; dAdo, 2'-deoxyadenosine; Ino, inosine; dThd, thymidine; Ura, uracil; Urd, uridine; dUrd, 2'-deoxyuridine; Rib, ribose. Formycin B (1 mM) and guanosine (0.22 mM) were also assessed (not shown): uridine fluxes in the presence of formycin B and guanosine were $>90\%$ and adenosine fluxes in the presence of formycin B and guanosine, respectively, were 50% and 60% of control values.

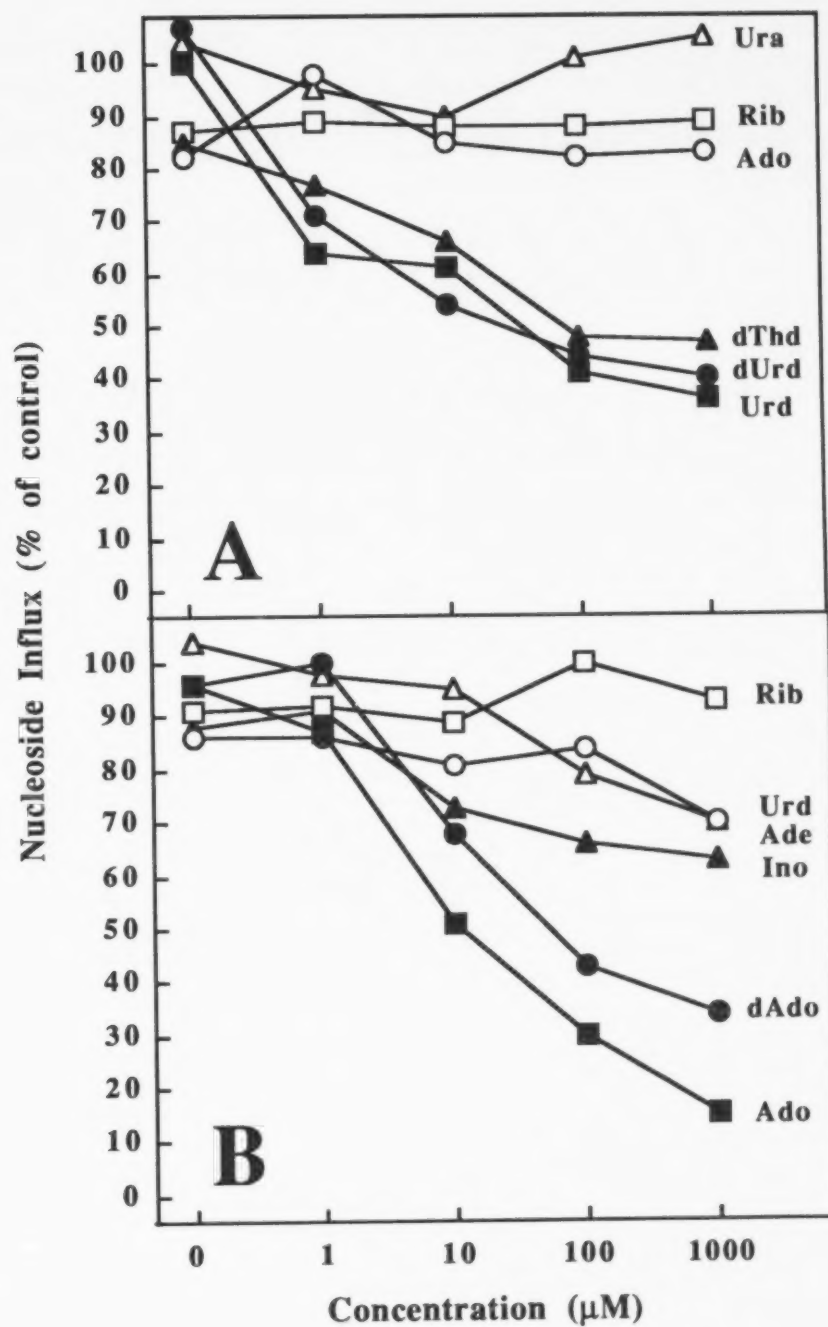


FIGURE V 5

The Low-Affinity Nucleoside Transport Process of Sf9 Cells Accepts Both Adenosine and Uridine as Substrates

Portions of Sf9 cells (100 μ l, 2×10^7 cells/ml) were incubated at RT ($\approx 22^\circ\text{C}$) with 100 μ l of BTB that contained 200 μM [^3H]uridine (A) or [^3H]adenosine (B) together with increasing concentrations of non-radioactive adenosine (■) or uridine (○). Influx assays, conducted in triplicate, were terminated after 0, 5, and 10 sec of incubation using the inhibitor-stop method and transport velocities were determined from time courses of influx by polynomial regression analysis using the least squares method. Influx (mean $\pm$ SD) in the presence of either adenosine or uridine are expressed as percent of flux in their absence (controls).

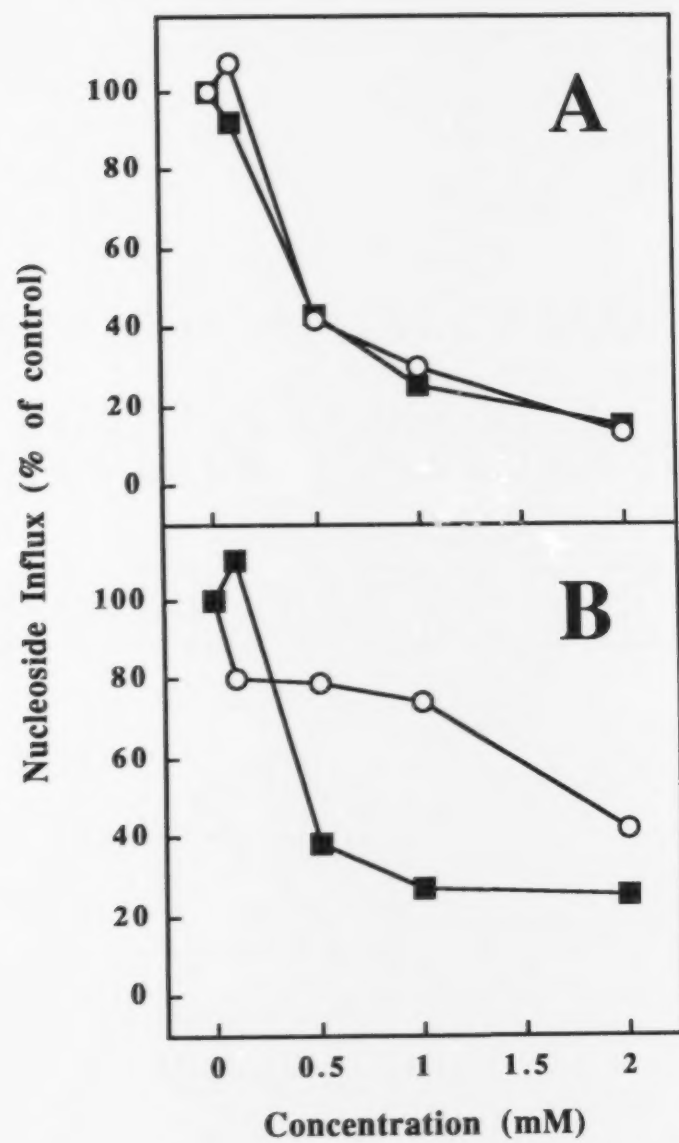


FIGURE V 6

High-Affinity Transport of Uridine and Adenosine is Insensitive to Inhibition by NBMPR, Dilazep and Dipyridamole

Sf9 cells were incubated (1.5×10^6 cells/ml) for 5 min at RT ($\approx 22^\circ\text{C}$) in BTB that contained graded concentrations of NBMPR (○), dilazep (■), or dipyridamole (●). Cells were harvested and portions ($100\ \mu\text{l}$, 1.5×10^7 cells/ml) were incubated for 10 sec, in the presence of the same inhibitor concentration, with $100\ \mu\text{l}$ of $2\ \mu\text{M}$ [^3H]uridine (A) or $2\ \mu\text{M}$ [^3H]adenosine (B), after which influx was terminated using the oil-stop method. Values (mean $\pm$ SD) obtained in the presence of inhibitors are expressed as a percent of values in the absence of inhibitor. Error bars are not shown where SD values were smaller than the size of data symbols.

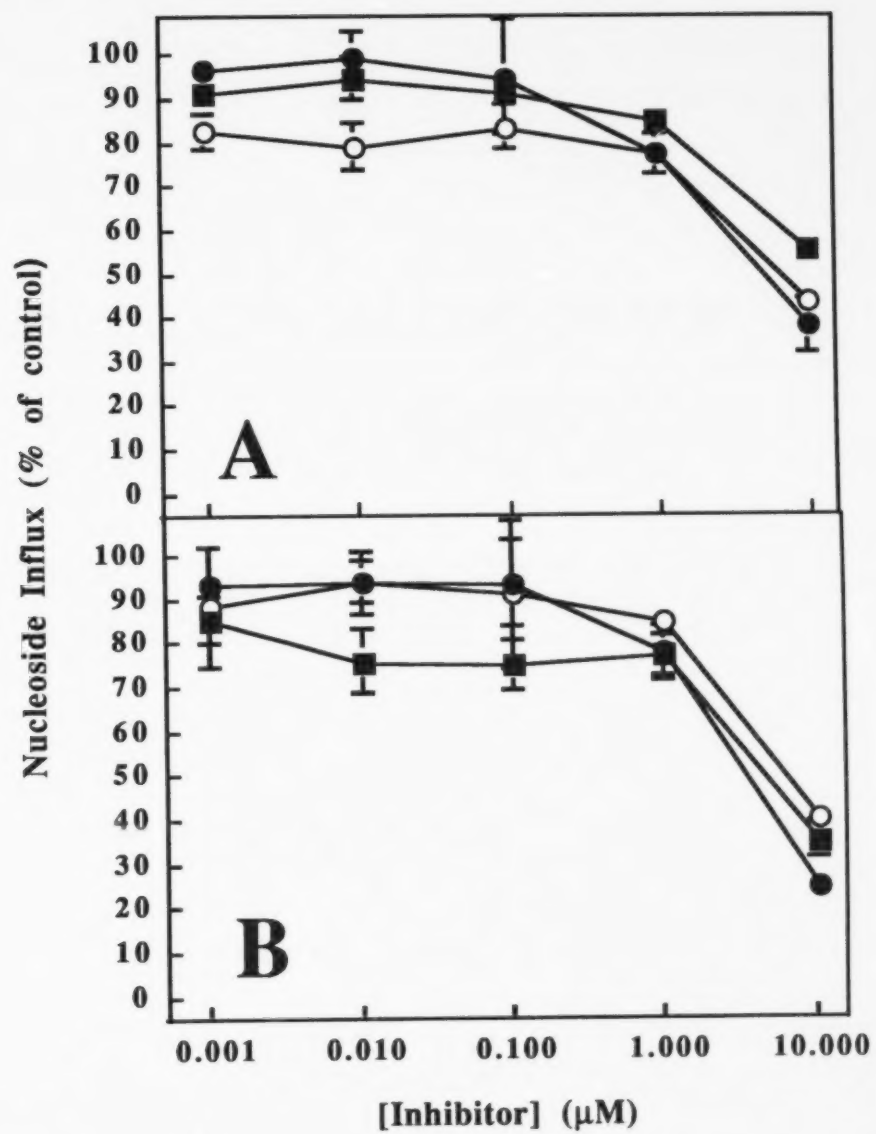


FIGURE V 7

Sf9 Cells Lack High-Affinity NBMPR Binding Sites

Actively proliferating Sf9 cells were incubated (2.0×10^6 cells/ml, RT, 20 min) in BTB containing graded concentrations of [^3H]NBMPR (0.5-5.0 nM) alone (●) or also containing 10 μM NBTGR (□). Portions of cell suspension (500 μl) were layered over 250 μl transport oil in microcentrifuge tubes and cells were pelleted by centrifugation. The cell-associated and actual free concentrations of [^3H]NBMPR were determined as described in Materials and Methods. Total bound [^3H]NBMPR (●) and non-specific binding (□) are mean values for triplicate determinations with SD of $\leq 3\%$ of the mean values. Results are from a single representative experiment.

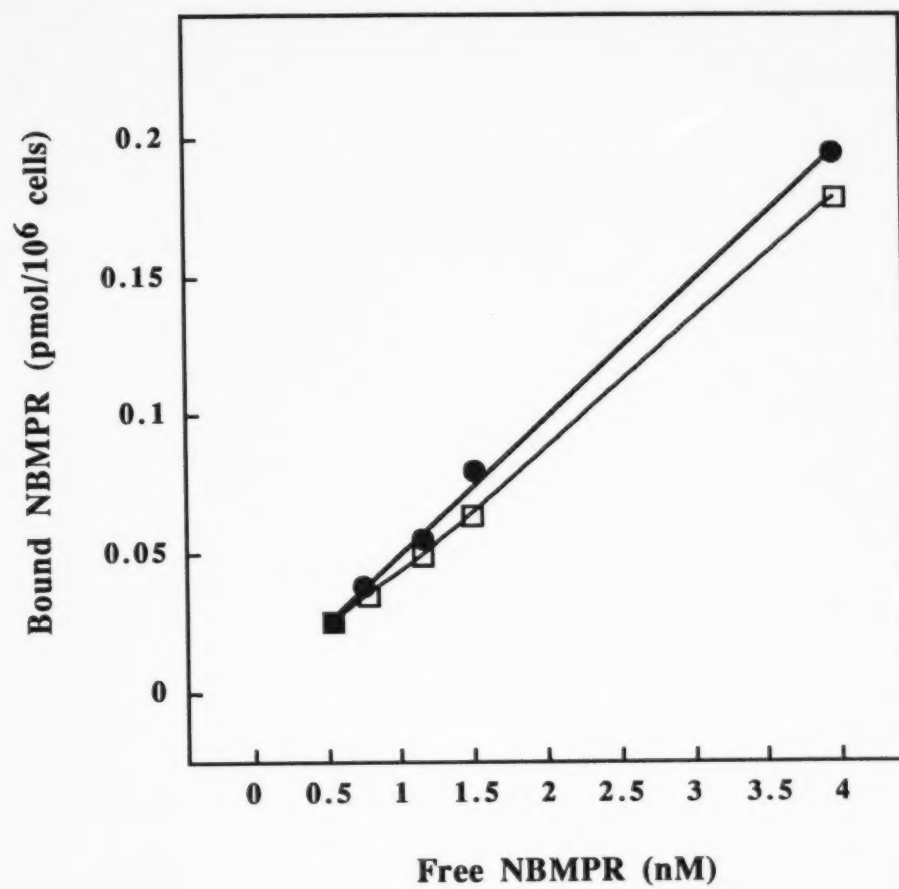


FIGURE V 8

Uptake of Nucleosides is Not Dependent on the Presence of Na⁺

Sf9 cells were harvested by centrifugation and washed once in BTB or Na⁺-free BTB, then resuspended at 1.5×10^7 cells/ml in the same buffer. Uptake of $10 \mu\text{M}$ [³H]uridine (A) and $10 \mu\text{M}$ [³H]adenosine (B) was determined at RT ($\approx 22^\circ\text{C}$) in the presence (O) or absence (□) of Na⁺ by the addition of cell suspension (2.0 ml) to either BTB or Na⁺-free BTB (2.0 ml) that contained $20 \mu\text{M}$ ³H-nucleoside. Portions of cell suspension (200 μl) were then layered over 200 μl of transport oil in microcentrifuge tubes after 0, 1, 2, 3, and 5 min of incubation and cells were pelleted by centrifugation. Values are the means $\pm$ SD of three determinations. Error bars are not shown where SD values were smaller than the size of data symbols.

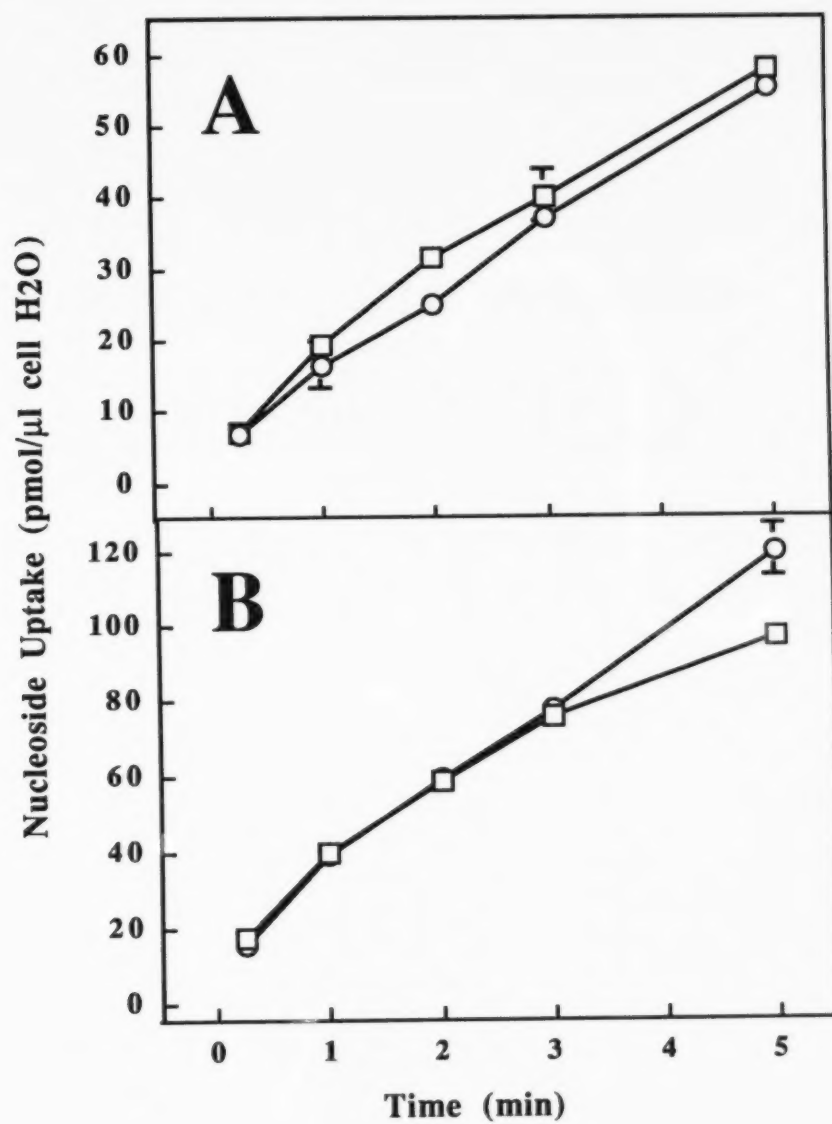
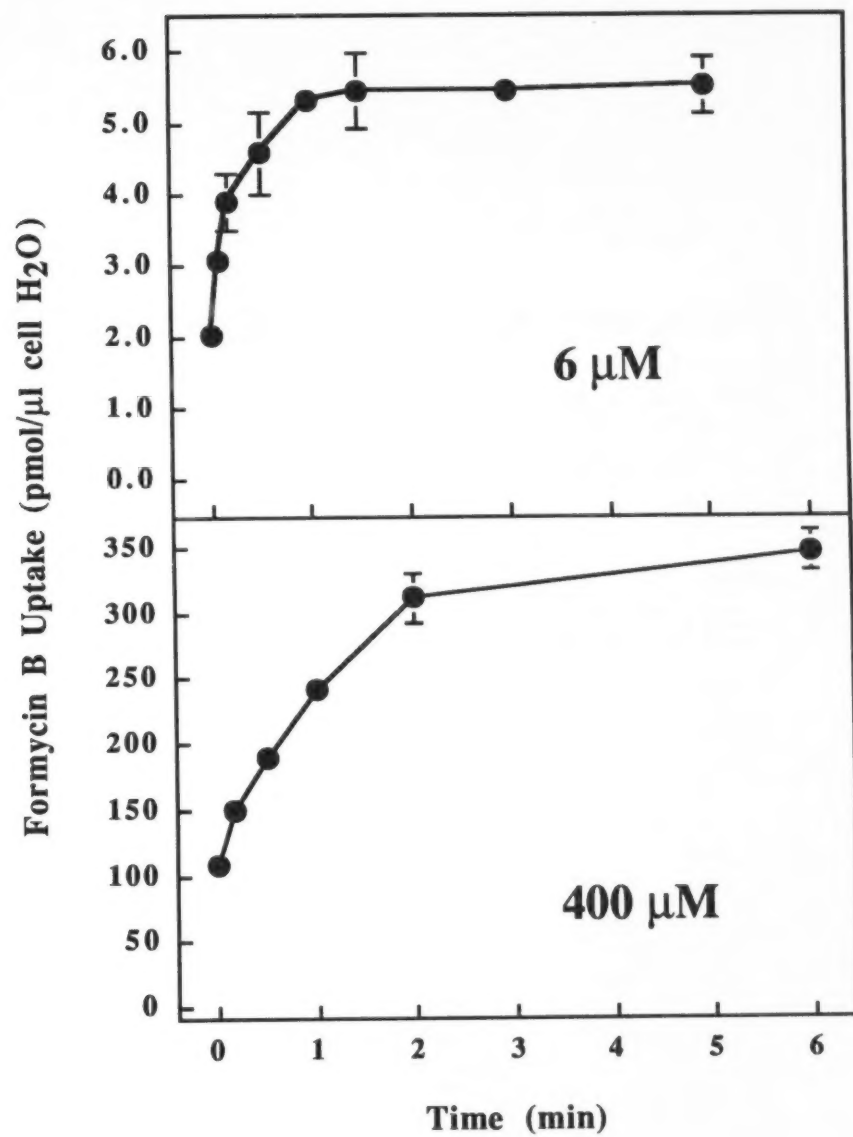


FIGURE V 9

Equilibrative Processes Mediate the Uptake of Formycin B

Suspensions of Sf9 cells ($1-1.5 \times 10^7$ cells/ml) were incubated at RT ($\approx 22^\circ\text{C}$) in BTB that contained $6\ \mu\text{M}$ or $400\ \mu\text{M}$ [^3H]formycin B. Portions of cell suspension were layered over $200\ \mu\text{l}$ of transport oil in microcentrifuge tubes and centrifuged at the indicated time intervals. Assays were conducted in triplicate. The apparent intracellular formycin B concentration (mean $\pm$ SD) was calculated assuming that $\text{pmol}/\mu\text{l}$ cell water equals μM . Error bars are not shown where SD values were smaller than the size of data symbols.



BIBLIOGRAPHY

- Abidi, T. F., Plagemann, P. G. W., Woffendin, C., and Stollar, V. (1987) Nucleoside and nucleobase transport and metabolism in wild type and nucleoside transport-deficient *Aedes albopictus* cells. *Biochim. Biophys. Acta.* **897**, 431-444.
- Aronow, B., Kaur, K., McCartan, K., and Ullman, B. (1987) Two high affinity nucleoside transporters in *Leishmania donovani*. *Mol. Biochem. Parasitol.* **22**, 29-37.
- Belt, J. A. (1992) Characterization of Na⁺-dependent, concentrative nucleoside transport of L1210 cells. *Proc. Amer. Assoc. Cancer Res.* **33**, 116.
- Boleti, B. (1991) Nucleoside transport in K562 leukemia cells. Ph.D. thesis, Department of Biochemistry, University of Alberta, Edmonton, Alberta.
- Carter, N. S. and Fairlamb, A. H. (1993) Arsenical-resistant trypanosomes lack an unusual adenosine transporter. *Nature* **361**, 173-175.
- Dagnino, L (1988) Sodium-driven nucleoside transport in mouse leukemia L1210 cells. Ph.D. thesis, Department of Pharmacology, University of Alberta, Edmonton, Alberta.
- Davey, R. A., Mayrhofer, G., and Ey, P. L. (1992) Identification of a broad-specificity nucleoside transporter with affinity for the sugar moiety in *Giardia intestinalis* trophozoites. *Biochim. Biophys. Acta.* **1109**, 172-178.
- Desai, S. A., Krogstad, D. J., and McCleskey, E. W. (1993) A nutrient-permeable channel on the erythrocytic malarial parasite. *Nature* **362**, 643 - 646.
- El Kouni, M. H. and Cha, S. (1987) Metabolism of adenosine analogues by *Schistosoma mansoni* and the effect of nucleoside transport inhibitors. *Biochem. Pharmacol.* **36**, 1099-1106.
- El Kouni, M. H., Diop, D., O'Shea, P., Carlisle, R., and Sommadossi, J. P. (1989) Prevention of tubercidin host toxicity by nitrobenzylthioinosine 5'-monophosphate for the treatment of schistosomiasis. *Antimicrob. Ag. Chemother.* **33**, 824-827.
- El Kouni, M. H., Messier, N. J., and Cha, S. (1987) Treatment of schistosomiasis by purine nucleoside analogues in combination with nucleoside transport inhibitors. *Biochem. Pharmacol.* **36**, 3815-3821.
- Ey, P. L., Davey, R. A., and Duffield, G. A. (1992) A low-affinity nucleobase transporter in the protozoan parasite *Giardia intestinalis*. *Biochim. Biophys. Acta.* **1109**, 179-186.
- Fincham, D. A., Wolowyk, M. W., and Young, J. D. (1991) Nucleoside transport by red blood cells from a primitive vertebrate, the pacific hagfish (*Eptatretus stouti*), is mediated by a nitrobenzylthioinosine-insensitive transport system. *Biochim. Biophys. Acta.* **1069**, 123-126.
- Finley, R. W., Cooney, D. A., and Dvorak, J. A. (1988) Nucleoside transport in *Trypanosoma cruzi*: analysis of a mutant resistant to tubercidin. *Mol. Biochem. Parasitol.* **31**, 133-140.

- Foury, F. and Goffeau, A. (1975) Stimulation of active uptake of nucleosides and amino acids by cyclic adenosine 3':5'-monophosphate in the yeast *Schizosaccharomyces cerevisiae*. *J. Biol. Chem.* **250**, 2354-2362.
- Gati W. P., Stoyke, A. F. W., Gero, A. M., and Paterson, A. R. P. (1987) Nucleoside permeation in mouse erythrocytes infected with *Plasmodium yoelii*. *Biochem. Biophys. Res. Comm.* **145**, 1134-1141.
- Gati, W. P., Dagnino, L., and Paterson, A. R. P. (1989) Enantiomeric selectivity of adenosine transport systems in mouse erythrocytes and L1210 cells. *Biochem. J.* **263**, 957-960.
- Gati, W. P., Lin, A. N., Wang, T. I., Young, J. D., and Paterson, A. R. P. (1990) Parasite-induced processes for adenosine permeation in mouse erythrocytes infected with the malarial parasite *Plasmodium yoelii*. *Biochem. J.* **272**, 277-280.
- Gero, A. M. (1989) Induction of nucleoside transport sites into the host cell membrane of *Babesia bovis* infected erythrocytes. *Mol. Biochem. Parasitol.* **35**, 269-276.
- Gero, A. M. and Upston, J. M. (1992) Altered membrane permeability: a new approach to malaria chemotherapy. *Parasitol. Today* **8**, 283-286.
- Gero, A. M., Bugledich, E. M. A., Paterson, A. R. P., and Jamieson, G. P. (1987) Stage-specific alteration of nucleoside membrane permeability and nitrobenzylthioinosine insensitivity in *Plasmodium falciparum* infected erythrocytes. *Mol. Biochem. Parasitol.* **27**, 159-170.
- Gero, A. M., Scott, H. V., O'Sullivan, W. J., and Christopherson, R. I. (1989) Antimalarial action of nitrobenzylthioinosine in combination with purine nucleoside antimetabolites. *Mol. Biochem. Parasitol.* **34**, 87-98.
- Grace, T.D.C. (1962) Establishment of four strains of cells from insect tissues *in vitro*. *Nature* **195**, 788-789.
- Grenson, M. (1969) The utilization of exogenous pyrimidines and the recycling of uridine-5'-phosphate derivatives in *Saccharomyces cerevisiae*, as studied by means of mutants affected in pyrimidine uptake and metabolism. *Eur. J. Biochem.* **11**, 249-260.
- Harris, D. I., Beechey, R. B., Linstead, D., and Barret, J. (1988) Nucleoside uptake in *Trichomonas vaginalis*. *Mol. Biochem. Parasitol.* **29**, 105-116.
- Hogue, D. L., Hodgson, K. C., and Cass, C. E. (1990) Effects of inhibition of N-linked glycosylation on nucleoside transport polypeptides of L1210 leukemia cells. *Biochem. Cell Biol.* **68**, 199-209.
- Hunston, D. L. (1975) Two techniques for evaluating small molecule-macromolecule binding in complex system. *Anal. Biochem.* **63**, 99-109.
- Jund, R., Weber, E., and Chevallier, M. R. (1988) Primary structure of the uracil transport protein of *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **171**, 233-251.
- Levy, M. G. and Read, C. P. (1975) Uptake of purine bases and nucleosides in *Schistosoma mansoni*. *J. Parasitol.* **61**, 627-632.

- Luckow, V. A. and Summers, M. D. (1988) Trends in the development of baculovirus expression vectors. *Bio/Technology* **6**, 47-55.
- Maeda, S. (1989) Expression of foreign genes in insects using baculovirus vectors. *Ann. Rev. Entomol.* **34**, 351-372.
- McCracken, R. O., Lumsden, R. D., and Page, C. R. (1975) Sodium-sensitive nucleoside transport by *Hymenolepis diminuta*. *J. Parasitol.* **61**, 999-1005.
- Odbunude, P. O. J., Ikediobi, C. O., and Baer, H. P. (1991) *Leishmania donovani*: characteristics of adenosine and inosine transporters in promastigotes of two different strains. *Expl. Parasitol.* **73**, 369-375.
- Ogbunude, P. O. J. and Baer, H. P. (1993) Nucleoside transport in parasites-current status and methodological aspects. *Int. J. Biochem.* **25**, 471-477.
- Page, C. R., and MacInnis, A. J. (1975) Characterization of nucleoside transport in *Hymenolepidid* cestodes. *J. Parasitol.* **61**, 281-290.
- Plagemann, P. G. W. and Woffendin, C. (1989) Use of formycin B as a general substrate for measuring facilitated nucleoside transport in mammalian cells. *Biochim. Biophys. Acta.* **1010**, 7-15.
- Rao, T. V. G., Verma, R.S., and Rajendra, P. (1983) Transport of purine, pyrimidine bases and nucleosides in *Candida albicans*, a pathogenic yeast. *Biochem. Int.* **6**, 409-417.
- Rosenthal, H.E. (1967) Graphic method for the determination and presentation of binding parameters in a complex system. *Anal. Biochem.* **20**, 525-532.
- Sherwood, A. and Stollar, V. (1982) *Aedes albopictus* cells resistant to adenosine because of a defect in nucleoside transport. *Somatics Cell Genet.* **8**, 575-585.
- Weber, E., Rodreguez, C., Chevallier, M. R., and Jund, R. (1990) The purine-cytosine permease gene of *Saccharomyces cerevisiae* : primary structure and deduced protein sequence of the FCY2 gene product. *Mol. Microbiol.* **4**, 585-596.

Chapter VI

GENERAL DISCUSSION

The aim of these studies was to elucidate important structural features of the transporter proteins that mediate the flux of nucleosides across the plasma membrane. To a large degree, this thesis has focused on developing methods for the identification of cDNAs that encode nucleoside transporters and upon the characterization of an isolated cDNA clone (*MTP*). These studies have also demonstrated the existence of previously uncharacterized nucleoside transporter processes in *S. cerevisiae* and *S. frugiperda* (Sf9) cells. Through this research, a greater awareness and understanding of previously undescribed classes of eucaryotic nucleoside transporters has been generated.

Why Are Plasma Membrane NT Proteins Glycosylated ?

It has become increasingly apparent that N-linked glycosylation of proteins is intimately linked to their proper expression at the plasma membrane. Recently, calnexin has been demonstrated to act as a chaperone in the endoplasmic reticulum (ER) by its ability to bind and promote the correct structural folding of newly synthesized glycoproteins via their N-linked core oligosaccharide moiety (Ou *et. al.*, 1993). A proposed model suggests that the absence of N-linked core oligosaccharide moieties normally present on glycoproteins, induced either by inhibition of core oligosaccharide synthesis (e.g., by tunicamycin treatment) or mutation of the signal sequence for N-linked glycosylation (Asn-X-Ser/Thr), results in the improper folding of many, but not all, newly synthesized glycoproteins (Hammond and Helenius, 1993). Implications of the inhibition of glycosylation in this model include (i) the retention of a non-folded protein in the ER and lack of its expression at the plasma membrane, (ii) the production and expression of an improperly folded protein at the plasma membrane, and (iii), in the case of a glycoprotein that does not require the activity of calnexin for proper folding, the expression of the properly folded protein at the plasma membrane.

The results of studies that examined the role of N-linked oligosaccharides in the functional expression of NT processes in L1210/C2 cells suggested that NT proteins are differentially sensitive to inhibition of N-linked glycosylation. Prolonged exposure of cells to low concentrations of tunicamycin did not decrease the numbers of NBMPR-binding sites in intact cells but did lead to a reduction in the amount of polypeptide in the plasma membrane that could be photolabeled with NBMPR, thereby suggesting that the lack of N-linked oligosaccharide moieties led to intracellular retention of *es* NT protein. However, the decrease in the K_m and V_{max} values of low-affinity uridine transport that were induced upon exposure of cells to tunicamycin over shorter periods was not easily explained. Whether these alterations were due to (i) the absence of N-linked oligosaccharides that were functionally important for transport, or (ii) to the incorrect folding of NT polypeptides could not be determined. To completely understand the role of N-linked oligosaccharides in NT protein function, the primary structure of these proteins must be known. Studies could then be performed with immunological probes which address the functional expression of a NT process within the context of the folded state of recombinant NT proteins that lack signal sequences for N-linked glycosylation. Alternatively, purified recombinant NT polypeptide could be enzymatically deglycosylated and its function examined by expression in proteoliposomes.

Thus, the question as to why NT proteins are glycosylated with N-linked oligosaccharide is only partially answered. The observation that inhibition of glycosylation induced the intracellular retention of the *es* NT protein of L1210/C2 cells suggests that this protein utilizes glycosylation as a tool for targeting itself for proper folding and expression in the plasma membrane. However, since Na^+ -dependent NT was significantly decreased and complex changes in the kinetic parameters of the low affinity transport activity were exhibited by tunicamycin-treated cells, it is likely that N-linked glycosylation also plays a role in the expression of proteins responsible for *ei* and *cif* NT.

The Applicability of a Method to Identify cDNA-Encoded Equilibrative NT Proteins by Functional Expression in Yeast

In the past decade there has been a veritable explosion in the identification of members of numerous protein families that mediate the transport of solutes across the plasma membrane of eucaryotic cells. The cDNA of many of these transporter proteins have been identified by functional expression cloning methods, however it should be noted that most of the identified transporters function in a concentrative manner. In particular, the use of functional expression screening methods in *X. laevis* oocytes to identify cDNA-encoded concentrative transporters is well documented and includes Na⁺-dependent glucose (Hediger *et al.*, 1987), nucleoside (Huang *et al.*, 1994) and amino acid transporters (Kanai and Hediger, 1992). However, only in a single case has a recombinant equilibrative (urea) transporter been identified by this method (You *et al.*, 1993).

Research in this thesis has produced a method by which recombinant NT proteins could be identified based upon their functional expression in *S. cerevisiae*. Since the expression of a recombinant NT protein by this method resulted in the survival and proliferation of a single transformed cell, the isolation of a cDNA that encoded a NT protein should have been straight forward. However, many "false positive" transformed cells that exhibited thymidine-dependent survival were identified, presumably due to higher levels of passive diffusion of thymidine across their plasma membranes.

In retrospect, a reduction in the numbers of these false positives could have been achieved by introducing minor modifications into the screening method. Recalling that the NT processes of mammalian cells accept thymidine, adenosine and uridine as permeants, one such modification could involve the use of KTK cells, which lack an adenosine influx process and contain adenosine phosphorylase (Laten *et al.*, 1986), exactly as described in Chapter III except that adenosine would be substituted for adenine in the culture medium. The survival of a transformed KTK cell would then be dependent upon the uptake of thymidine and adenosine to complement its depletion of intracellular dTMP and adenine auxotrophy, respectively. The likelihood of producing a false positive cell with increased passive diffusion of both purine and pyrimidine nucleosides may be lower than for a single nucleoside.

The functional expression screening method in yeast was used to identify a mammalian NT protein (*MTP*), however, this NT protein was apparently not similar to proteins that mediate NT across the plasma membrane of mammalian cells. The inability of this method to identify cDNA of these NT proteins may have been due to unforeseen circumstances, such as the inability to properly process or express a mammalian NT protein in the yeast plasma membrane. Therefore, the "acid test" for this proposed method will likely involve the functional expression of an isolated cDNA-encoded NT protein in KTK cells under the conditions of the screening method. Unfortunately, this was the very reason for the creation of this screening method.

Intracellular Nucleoside Transporters

Research in the field of mammalian NT has primarily focused upon the plasma membrane transport processes and has, for the most part, not addressed the possibility of the existence of mediated NT across the membranes of intracellular compartments. Exceptions to this general statement include the presence of poorly characterized NT processes in lysosomes (Pisoni and Thoene, 1989) and mitochondria (Mitra and Bernstein, 1970; Walker and Lewis, 1987). Thus, the identification of *MTP* as a putative mammalian Golgi-specific NT protein during this research was unexpected. At least eight functionally distinct nucleotide/nucleotide-sugar transport processes mediate the coupled influx and efflux of nucleotide-sugars and nucleotide, respectively, across the Golgi membrane (for review, see Hirschberg and Snider, 1987). The sugar of the luminal nucleotide-sugars are transferred to oligosaccharide moieties of glycoproteins in

transferase-mediated reactions that yield free nucleotides. The nucleotide base of the permeant is recognized by the transporter and the transport of pyrimidine nucleotide-sugars is inhibited by only pyrimidine nucleotides whereas that of purine nucleotide-sugars is inhibited by only purine nucleotides. Acceptance as a permeant generally requires the presence of a 5'-phosphate, however, a uridine-sugar conjugate has been shown to inhibit one, or more, of these transport processes (Harvey and Thomas, 1993). The transport of UDP-*N*-acetylglucosamine and UDP-glucose into the lumen of the rough endoplasmic reticulum is also mediated by similar nucleotide/nucleotide-sugar transport processes (Hirschberg and Snider, 1987).

MTP accepts nucleosides as permeants and its' uptake of thymidine was inhibited by dTMP, but its overall permeant selectivity was not addressed. Future studies that examine if nucleoside- or nucleotide-sugars are permeants of *MTP* should determine whether this transporter represents one of the nucleotide/nucleotide-sugar transport processes present in the Golgi membrane. The northern blot analyses conducted with a *MTP* cDNA probe revealed the presence of more than one distinct hybridization signal in RNA from cultured cells and mouse tissues, an observation that was initially attributed to RNA degradation. If *MTP* does represent one of the Golgi nucleotide/nucleotide-sugar transporters, these multiple hybridization signals may actually be due to the presence of RNA transcripts of other related transporters. Alternatively, *MTP* may represent a previously undescribed NT process present in the membranes of an intracellular compartment.

Functional Dissimilarity Between Plasma Membrane NT Processes of Eucaryotic Cells

This research has also characterized NT processes present in the plasma membranes of *S. cerevisiae*, and murine L1210/C2 and *Spodoptera frugiperda* cells. The L1210/C2 cells expressed equilibrative and concentrative NT processes typically found in many other mammalian tissues and cultured cells. These mammalian NT processes exhibit differences in inhibitor sensitivity, Na⁺-dependence, kinetic parameters, and general permeant selectivity, however, all these processes share an overlapping permeant selectivity for adenosine and uridine. This one common feature suggests that their permeant recognition sites are, to an extent, similar. Since most non-mammalian cells also exhibit an overlapping permeant selectivity for adenosine and uridine (For review, see the Introduction of Chapter V), this similarity of permeant recognition site appears to be "conserved" between most eucaryotic cells. However, the uridine transport process of *S. cerevisiae* and the high-affinity purine- and pyrimidine-selective NT processes of *S. frugiperda* cells do not exhibit any overlapping permeant selectivity for adenosine and uridine. In fact, the specificity of the transport process in *S. cerevisiae* is such that thymidine and cytidine are not recognized. These observations would suggest that the permeant recognition sites of these nucleoside transporters are fundamentally dissimilar in structure to that of most other eucaryotic nucleoside transporters. Such predictions are purely hypothetical when based upon functionally-derived results, therefore confirmation of this proposed basic structural difference awaits the generation of information concerning the amino acid sequences and predicted structures of these nucleoside transporter proteins.

BIBLIOGRAPHY

- Hammond, C. and Helenius, A. (1993) A chaperone with a sweet tooth. *Curr. Biol.* 3, 884-886.

- Hediger, M. A., Coady, M. J., Ikeda, T. S., and Wright, E. M. (1987) Expression cloning and cDNA sequencing of the Na⁺/glucose co-transporter. *Nature* **330**, 379-381.
- Huang, Q. Q., Yao, S. Y. M., Ritzel, M. W. L., Paterson, A. R. P., Cass, C. E., and Young J. D. (1994) Cloning and functional expression of a complementary DNA encoding a mammalian nucleoside transporter protein. Submitted for Publication.
- Kanai, Y. and Hediger, M. A. (1992) Primary structure and functional characterization of a high-affinity glutamate transporter. *Nature* **360**, 467-471.
- Laten H. M., Valentine, P. J., and Van Kast, C. A. (1986) Adenosine accumulation in *Saccharomyces cerevisiae* cultured in medium containing low levels of adenine. *J. Bacteriol.* **166**, 763-768.
- Mitra, R. S. and Bernstein, I. A. (1970) Thymidine incorporation into deoxyribonucleic acid by isolated rat liver mitochondria. *J. Biol. Chem.* **245**, 1255-1260.
- Ou, W. J., Cameron, P. H., Thomas, D. Y., and Bergeron, J. J. (1993) Association of folding intermediates of glycoproteins with calnexin during protein maturation. *Nature* **364**, 771-776.
- Pisoni, R. L. and Thoene, J. G. (1989) Detection and characterization of a nucleoside transport system in human fibroblast lysosomes. *J. Biol. Chem.* **264**, 4850-4856.
- Walker, L. F. and Lewis, R. A. (1987) The metabolism of deoxyguanosine in mitochondria. Characterization of the uptake process. *Mol. Cell. Biochem.* **77**, 71-77.
- You, G., Smith, C. P., Kanai, Y., Lee, W. S., Steizner, M., and Hediger, M. A. (1993) Cloning and characterization of the vasopressin-regulated urea transporter. *Nature* **365**, 844-847.

Appendix A

A METHOD FOR IDENTIFICATION OF NBMPR-INSENSITIVE NUCLEOSIDE TRANSPORTERS BY EXPRESSION CLONING IN Cos-1 CELLS

Expression cloning in mammalian cells, originally described by Wong *et al.* (1985) and Seed and Aruffo (1987), has proven useful in the identification of numerous cDNAs encoding cell surface receptors and secreted proteins. The use of Cos-1 cells as a potential host cell type in a functional expression cloning approach to identify mammalian nucleoside transporter cDNA was examined during the course of my studies. Since most cultured mammalian cell types simultaneously express multiple nucleoside transport (NT) processes, expression cloning with such cell types would fail because of the presence of NT activity unless the NT processes could be eliminated genetically or pharmacologically. I have developed a method by which a single Cos-1 cell expressing a recombinant NBMPR-insensitive NT protein could be identified based on its ability to concentrate [^3H]nucleoside in the presence of NBMPR.

The nature of the process(es) that mediate the uptake of uridine and adenosine into Cos-1 cells was addressed by measuring influx of 25 μM [^3H]nucleoside over a long time period. As shown in Fig. A1, uridine and adenosine were rapidly accumulated by Cos-1 cells over a period of 5 min. Accumulation of both adenosine and uridine was completely inhibited in the presence of 10 μM NBMPR, suggesting the presence of only NBMPR-sensitive NT processes in the plasma membrane of Cos-1 cells. From these studies, it was estimated that a single Cos-1 cell expressing an NBMPR-insensitive NT process (either *ci* or *ei* type process) would be able to concentrate [^3H]nucleoside due to metabolic trapping and create 6-10 fold higher concentrations of intracellular [^3H]nucleoside in the presence of NBMPR than a Cos-1 cell expressing only its native NBMPR-sensitive NT process, due . To exploit a difference in NT capacities between cells for expression cloning, it was necessary to have a method to identify single cells that express a NBMPR-insensitive NT process.

Several studies have utilized autoradiography, based on cell surface binding of [^{125}I]ligand, to identify cDNA-encoded plasma membrane receptors in single Cos cells (Gearing *et al.*, 1989; Sasaki *et al.*, 1991; McMahan *et al.*, 1991). To determine if a single cell expressing a NBMPR-insensitive NT process could be detected by a similar approach, Cos-1 and HeLa cells were grown together in mixed cultures on tissue culture inserts and long-term uptake of 10 μM [^3H]adenosine in the presence of 25 μM NBMPR was measured by autoradiography. HeLa cells have been demonstrated to express both the *es* and *ei* NT processes (Lauzon and Paterson, 1977), and as such, should exhibit a "NT phenotype" similar to that expected in a Cos-1 cell expressing a recombinant NBMPR-insensitive NT protein. The results of one such experiment, as shown in low-magnification micrographs, are illustrated in Fig. A2. The capacity of individual HeLa cells to accumulate intracellular [^3H]adenosine was evident by the appearance of foci silver grains (Panel A, Fig. A2). There were no similar foci in cultures of Cos-1 cells grown under identical conditions, although a background of silver grains was present (Panel B, Fig. A2). In contrast, individual foci of silver grains were seen in ~50% confluent cultures of Cos-1 cells that had been seeded with approximately 50 HeLa cells (Panel C, Fig. A2).

From the results shown in Fig. A2, the theoretical limit of detection of this method should be at least as low as 1 NBMPR-insensitive NT-expressing cell per tissue culture insert at ~50% cell confluency (about 0.5×10^6 Cos-1 cells). Expression of recombinant proteins screening in Cos cells typically employs a DEAE-dextran method of transient transfection method (Ausubel *et al.*, 1989). Since efficiencies of transient transfection of 50% are not uncommon with Cos cells, approximately 0.6×10^5

independent library cDNA clones could be transfected into 1.25×10^5 Cos cells and plated onto a single culture insert. The transfected cell population would be expected to grow to $\approx 0.5 \times 10^6$ cells after a period of 2 days, whereupon expression screening would be conducted. Therefore, a cDNA expression library of 2×10^6 independent clones could be screened using a total of 10 independent transfection reactions plated onto separate inserts.

In conclusion, this investigation has established a potentially powerful method for identification of cDNA that encode NBMPR-insensitive NT proteins of mammalian cells. Hopefully, the use of this method will expedite the identification of equilibrative and concentrative NT proteins and generate information concerning the functional and structural relationships between classes of NT proteins.

FIGURE A 1

Cos-1 Cells Are Unable to Internalize Adenosine or Uridine in the Presence of NBMPR

Cos-1 cells (ATCC, CRL #1651) were grown to approximately 50% confluency on 35-mm tissue culture dishes (Lux Scientific) in DMEM medium supplemented with NaHCO_3 (2 g/l) and 10% FBS. Cell cultures were incubated (15 min, RT) with transport buffer (1.0 mM CaCl_2 , 2.6 mM KCl, 1.4 mM KH_2PO_4 , 0.5 mM MgCl_2 , 5.0 mM glucose, 138.0 mM NaCl, 8.0 mM Na_2HPO_4 , pH 7.4) in the absence ($\circ$, $\square$) or presence ($\bullet$, $\blacksquare$) of 10 μM NBMPR. The incubation solution was removed and a 1.0-ml volume of transport buffer containing 25 μM [^3H]adenosine ($\circ$, $\bullet$) or [^3H]uridine ($\square$, $\blacksquare$), was added. In the case of cells pre-incubated with NBMPR, the 25 μM [^3H]-nucleoside solution also contained 10 μM NBMPR. After various periods of incubation at RT, the [^3H]nucleoside-containing buffer was aspirated and culture dishes were rapidly washed twice in large volumes of ice-cold transport buffer that contained 100 μM dilazep. Cells were solubilized in 0.5 ml of 5% (v/v) Triton X-100 and combined with 5.0 ml of EcoLite scintillant for measurement of radioactivity. Assays were conducted in triplicate and values presented are means $\pm$ SD. Shown in the insert are results from an experiment conducted with 25 μM [^3H]uridine on a different day.

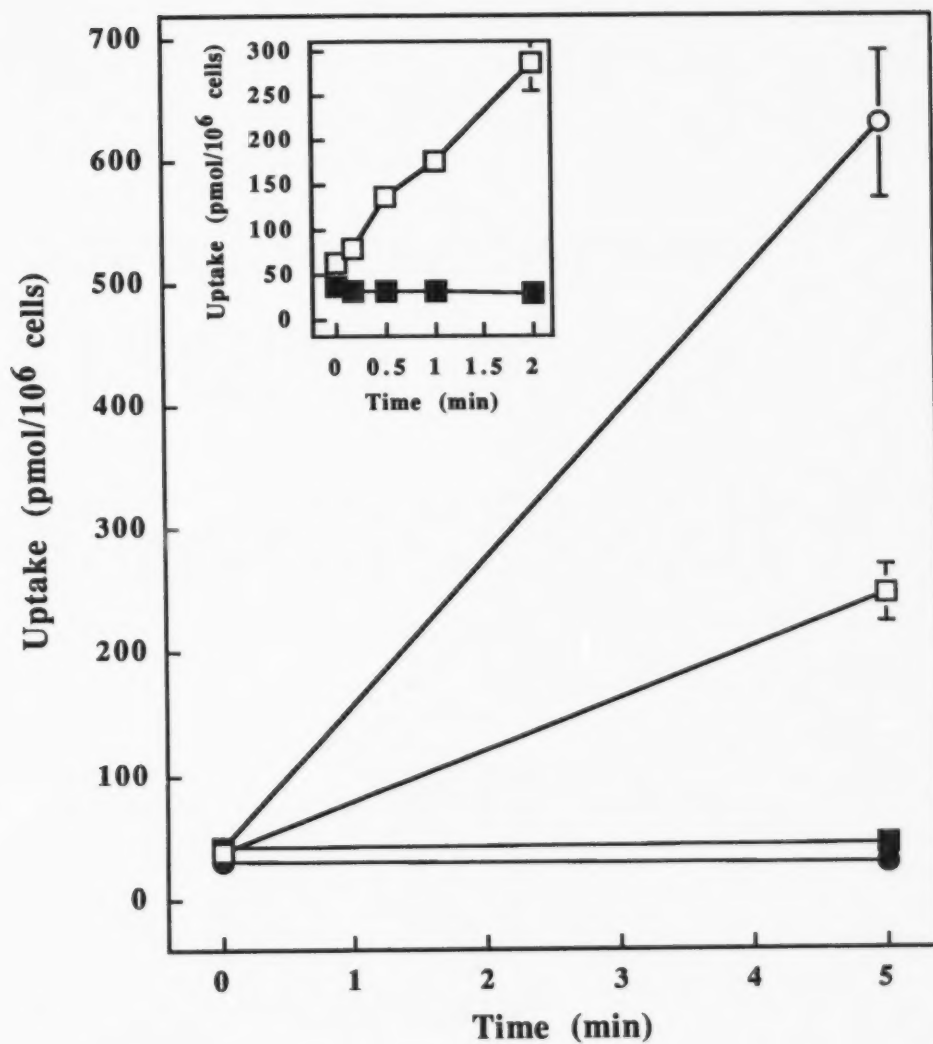
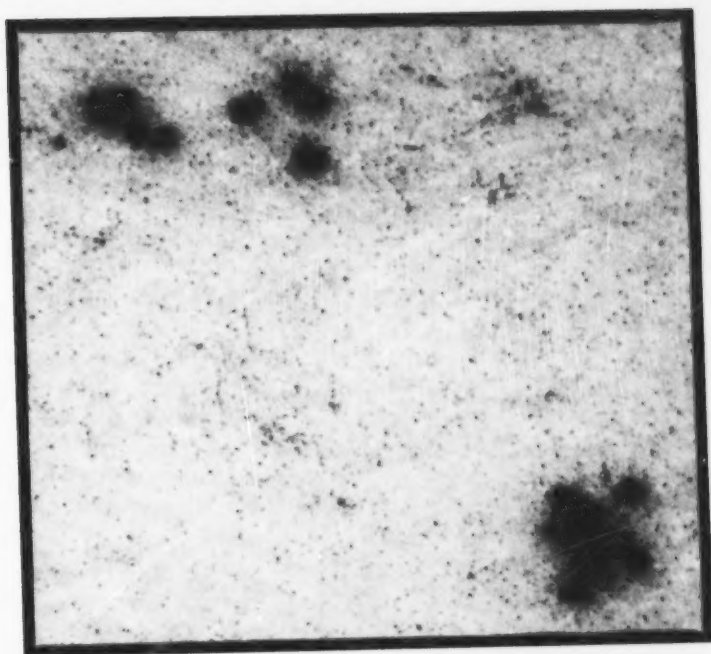


FIGURE A 2

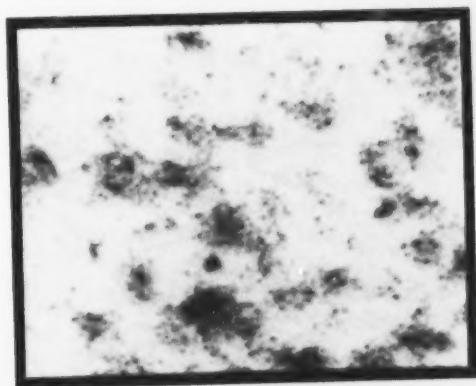
Detection of Single Cells Expressing a NBMPR-Insensitive NT Process by Autoradiography

Actively proliferating cultures of Cos-1 and Hela S3 (ATCC, CRL #2.2) cells were trypsinized and replated in Nunc TC 25 inserts as either; 50 individual HeLa cells alone (**Panel A**), a subconfluent ($\approx 0.5 \times 10^6$ cells/insert) culture of Cos-1 cells alone (**Panel B**), or a mixture of 50 individual HeLa cells and a subconfluent culture of Cos-1 cells (**Panel C**). Cultures were grown for 12 hr in normal medium and then incubated (20 min, 37°C) in DMEM medium supplemented with NaHCO_3 (2 g/l), 1% FBS, 1 μM deoxycoformycin, and 25 μM NBMPR. The incubation medium was removed and the TC inserts were incubated (6 hr, 37°C) in a 1.0-ml volume of fresh incubation medium that contained [2,8- ^3H]adenosine (approximate final concentrations: 10 μM , 2×10^5 cpm/ μl). The [^3H]nucleoside-containing buffer was aspirated and the inserts were rapidly washed twice in large volumes of ice-cold transport buffer that contained 100 μM dilazep and then were allowed to dry at RT. The inserts were punched out of their supports and placed, culture side facing upwards, in a film cassette. Amersham [^3H]-Hyperfilm was placed, emulsion side facing downwards, on top of inserts and incubated at -70°C for 4-7 days. The film was developed manually according to the manufacturer's instructions.

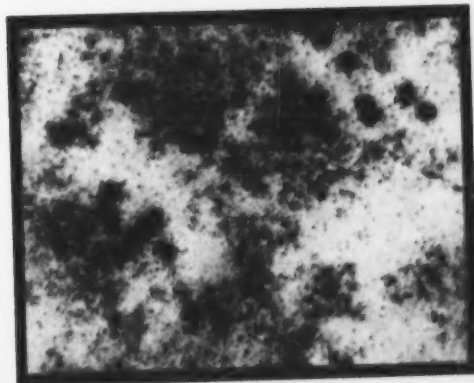
A.



B.



C.



Bibliography

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1989) Current protocols in molecular biology.

Gearing, D. P., King, J. A., Gough, N. M., and Nicola, N. A. (1989) Expression cloning of a receptor for human granulocyte-macrophage colony-stimulating factor. *EMBO J.* **8**, 3667-3676.

Lauzon, G. J. and Paterson, A. R. P. (1977) Binding of the nucleoside transport inhibitor nitrobenzylthioinosine to HeLa cells. *Mol. Pharmacol.* **13**, 883-891.

McMahan, C. J., Slack, J. L., Mosley, B., Cosman, D., Lupton, S. D., Brunton, L. L., Grubin, C. E., Wignall, J. M., Jenkins, N. A., Brannin, C. I., Copeland, N. G., Huebner, K., Croce, N. G., Cannizzarro, L. A., Benjamin, D., Dower, S. K., Spriggs, M. K., and Sims, J. E. (1991) A novel IL-1 receptor, cloned from B cells by mammalian expression, is expressed in many cell types. *EMBO J.* **10**, 2821-2832.

Sasaki, K., Yamano, Y., Bardhan, S., Iwai, N., Murray, J. J., Hasegawa, M., Matsuda, Y., and Inagami, T. (1991) Cloning and expression of a complementary cDNA encoding a bovine adrenal angiotensin type-1 receptor. *Nature* **351**, 230-232.

Seed, B. and Aruffo, A. (1987) Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. *Proc. Natl. Acad. Sci. USA* **84**, 3365-3369.

Wong, G. G., Witek, J. S., Temple, P. A., Wilkens, K. M., Leary, A. C., Luxenberg, D. P., Jones, S. S., Brown, E. L., Kay, R. M., Orr, E. C., Shoemaker, C., Golde, D. W., Kaufman, R. J., Hewick, R. M., Wang, E. A., and Clark, S. C. (1985) Human GM-CSF: Molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. *Science* **228**, 810-815.